REMARKS

Entry of the amendment is requested, whereby claims 38-58, 60, 61, and 63-66 will remain pending. Claims 38-41, 43-44, 58, and 60 are amended.

The amendments, inter alia, make changes directed to the Examiner's objections.

The remaining changes now require full hybridization and a specific, functional nucleotide sequence.

At page 6, line 23, application Serial Number 938 334 is incorporated by reference. This application is now U.S. Patent No. 5,405,940. For convenience, a copy of the front page of this patent is attached. This will show that the '940 patent describes MAGE-4 and MAGE-41 based tumor rejection antigens, and describes the sequence which encodes these. This sequence corresponds to those set forth by amendment in the claims. Hence, the amendment is supported by the specification, is proper, and should be entered.

The Examiner has rejected all claims as allegedly not satisfying the written description requirement. Similarly, the Examiner has rejected all claims under 35 U.S.C. § 112, first paragraph, as alleging that the claims are not enabled. Both rejections are traversed.

The Examiner argues that because Brasseur et al., *Int. J. Cancer*, 52:839-841 (1992) (which is not prior art), teaches that Northern Blotting did not suffice to distinguish various MAGE family members, PCR did. Therefore, the claims lack written description.

This rejection makes no sense. The art relied upon shows that while one method may have failed, another worked. Hence, the sequence can be distinguished, one from the other. Further, the claims now require a specific sequence NOT found in other MAGE molecules. Since PCR can distinguish the molecules, it is a matter of routine skill in the art to develop relevant, MAGE-4 and MAGE-41 specific primers, based upon this sequence.

With respect to the argument set forth at page 6, again this is not understood. The specification takes pains to explain how the invention differs from TSTA molecules, and hence this is irrelevant.

With respect to the argument presented over pages 7 and 8, the Examiner appears to be requiring structural characteristics. As was pointed out, supra, the claims require a sequence unique to the MAGE-4 family, and satisfy the alleged requirement.

Similarly, it is believed the enablement rejection cannot be maintained.

First of all, it has been shown that MAGE-4 and MAGE-41 molecules are processed to tumor rejection antigens, and the claims expressly recite such sequences. Other tumor rejection antigens are produced by MAGE-4 and MAGE-41 molecules, as is shown by the attached papers. While these are not prior art, if the Examiner is going to rely on non-prior art reference, so can applicants.

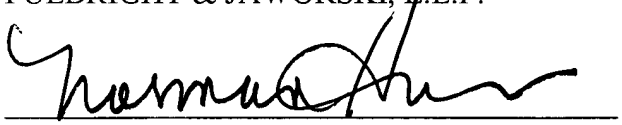
The arguments over pages 12-17 is believed to be irrelevant to the pending claims. None of this discussion deals with MAGE-4 or MAGE-41.

Further, since the claims expressly require the presence of a known and defined tumor rejection antigen, the references, to the extent they are prior art (and most of them are not), are irrelevant.

It is believed this application is in condition for allowance. If it is not, an interview is requested.

Respectfully submitted,

FULBRIGHT & JAWORSKI, L.L.P.



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Enclosures: Front Page of US Patent No. 5,405,940
Papers showing MAGE-4 and MAGE-41 Molecules

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New MAGE-4 antigenic peptide recognized by cytolytic T lymphocytes on HLA-A1 tumor cells

Key words:
CTL; HLA-A1; *MAGE-4*; peptide; tetramer; tumor

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We thank Sébastien Loeuille for expert technical assistance and comments. We thank Pierre Coulie for critical reading and Mrs Nathalie Krack for editorial assistance. Christophe Lonchay was supported by the TELEVIE fund (Belgium). This work was supported by the Belgian Programme on Interuniversity Poles of Attraction initiated by the Belgian State, Prime Minister's Office, Science Policy Programming, by a grant from the Fédération Belge Contre le Cancer (Belgium), and by grant no. QLK3-CT-1999–00064 from the European Community, Fifth Framework programme.

Abstract: 'Cancer-germline' genes such as those of the *MAGE* family are expressed in many tumors and in male germline cells, but are silent in other normal tissues. They encode shared tumor-specific antigens, which have been used in small therapeutic vaccination trials of cancer patients. Gene *MAGE-4*, which is expressed in more than 50% of carcinomas of esophagus, head and neck, lung, and bladder, has two known alleles. Using PCR amplifications and digestions of the amplified product, we found that one third of the *MAGE-4*-positive samples expressed *MAGE-4a*. We folded HLA-A1 tetramers with peptide *MAGE-4a*$_{169-177}$ EVDPASNTY, which is homologous to *MAGE-1*- and *MAGE-3*-encoded peptides recognized on HLA-A1 by cytolytic T lymphocytes. Blood lymphocytes from an individual without cancer were directly labelled with these A1/*MAGE-4* tetramers. The very rare cells that were stained were sorted by flow cytometry and cloned. We isolated a cytolytic T-lymphocyte clone that lyzed specifically cells pulsed with this *MAGE-4* peptide and HLA-A1 tumor cells expressing *MAGE-4a*, demonstrating that this antigenic peptide is processed efficiently in tumor cells. This peptide might therefore be useful for therapeutic antitumoral vaccination.

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The *MAGE* gene family comprises 24 functional genes divided into three clusters, named *MAGE-A*, *B*, and *C* (1–4). These genes are expressed in many human tumors of different histological types, but are silent in normal cells with the exception of male germline cells. Some of them are also expressed in placenta, including *MAGE-A4*, which will be referred to hereafter as *MAGE-4* (1). Male germline cells and placenta do not express MHC class I molecules and therefore cannot present antigens to cytolytic T lymphocytes (CTLs) (5). *MAGE*-encoded antigens are thus tumor-specific and of particular interest for cancer immunotherapy, as they are shared by many

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tumors. Clinical trials involving *MAGE* antigens have been performed in melanoma patients, and tumor regressions have been observed in a minority of patients (6–11).

There are two known alleles of gene *MAGE-4*: *MAGE-4a* and *MAGE-4b*. The putative proteins differ by a single amino acid (12). The distribution of the two alleles in the population is unknown. The gene contains eight alternative first exons that are spliced to unique second and third exons. The entire open reading frame is located in the third exon.

We reported previously the identification of two *MAGE-4* antigenic peptides recognized by CTLs: GVYDGREHTV ($MAGE\text{-}4_{230-239}$) presented by HLA-A2 and SESLKMIF ($MAGE\text{-}4_{156-163}$) presented by HLA-B37 (13, 14). Both peptides are encoded by each of the two *MAGE-4* alleles. To produce these anti-MAGE-4 CTLs, we had stimulated CD8 T cells from non-cancerous blood donors with autologous dendritic cells infected with adenoviruses or poxviruses, carrying the coding sequence of *MAGE-4*. Here, we have used a different strategy: a large number of blood lymphocytes were directly labelled with HLA-peptide tetramers folded with a *MAGE-4a*-encoded peptide. The very rare cells that were stained were sorted by flow cytometry, amplified in clonal conditions and analyzed further for tetramer labelling and specific lytic activity. We describe here the isolation of a CTL clone directed against a new *MAGE-4a* antigen.

Materials and methods

Cell lines, media, and reagents

The Epstein-Barr virus-transformed B (EBV-B) cell lines and the tumor cell lines were cultured in IMDM (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Life Technologies). COS-7 cells were maintained in DMEM (Life Technologies) supplemented with 5% fetal calf serum. All the media were supplemented with 0.24 mM of L-asparagine, 0.55 mM of L-arginine, 1.5 mM of L-glutamine (AAG), 100 U/ml of penicillin and 100 μg/ml of streptomycin. Human recombinant IL-2 was purchased from Eurocetus (Amsterdam, the Netherlands), IL-7 from Peprotech (Rocky Hill, NJ), and GM-CSF (Leucomax) from Schering-Plough (Brinny, Ireland). Human recombinant IL-4, IL-6, and IL-12 were produced in our laboratory. One U/ml of IL-6 is the concentration needed to obtain half-maximal proliferation of mouse 7TD1 cells (15). Geneticin was purchased from Life Technologies. Cell line MZ2-MEL. 2.2-*MAGE-4* was obtained by cotransfecting into MZ2-MEL. 2.2, a pcDNAI/Amp plasmid (Invitrogen) that contained the coding sequence of *MAGE-4a* together with vector pSVtkneoβ, which contained the coding sequence conferring resistance to geneticin (14). CTL ROLE 521/A10, which is specific for influenza basic polymerase 1 peptide VSDGGPNLY, was obtained after stimulation of blood cells with influenza basic polymerase 1 peptide VSDGGPNLY and sorting by flow cytometry after staining with influenza PB1.A1 tetramer.

Tetramer production and labelling with tetramers

Recombinant HLA-A1 molecules were folded *in vitro* with β2-microglobulin and peptide EVDPASNTY from MAGE-4a, or peptide VSDGGPNLY from the influenza basic polymerase 1. They were purified by gel filtration, biotinylated, and mixed as described (16) with Extravidin-PE (Sigma, St Louis, MT) for the HLA-A1/*MAGE-4a* tetramer, or streptavidin-APC (Molecular Probes, Eugene, OR) for the influenza control tetramer. For staining, cells were washed, resuspended at 20×10^6 cells per ml in PBS with 1% HS and incubated for 15 min at 4°C with HLA-A1 tetramers loaded with the MAGE-4a peptide (60 nM) or influenza peptide (20 nM). Anti-CD8 antibodies coupled to FITC (SK1 at 1/50, BD-Pharmingen, San Diego, CA), were added and after a further incubation for 15 min the cells were washed.

MACS and FACS sorting

Tetramer-labelled cells (10^7 cells/80 μl) were incubated with anti-PE microbeads (20 μl) according to the instructions of the manufacturer (Miltenyi Biotec, Bergish Gladbach, Germany), washed and sorted through a separation column inserted to a magnet in an AUTOMACS™ at 0.5 ml/min (Miltenyi Biotec). FACSVantage™ cell sorter (BD-Pharmingen) was used for FACS sorting at 1 cell per microwell and FACSCalibur™ (BD-Pharmingen) for analysis of the T-cell clones, using Cellquest™ software (BD-Pharmingen).

Culture conditions of cells sorted by flow cytometry

Sorted cells were cultured in U-bottomed microwells in 200 μl of IMDM supplemented with AAG, 10% human serum, IL-2 (100 U/ml), IL-4 (5 U/ml), and IL-7 (5 ng/ml). They were stimulated every 9–10 days with 1×10^3 irradiated (100 Gray) autologous peptide-pulsed dendritic cells, in the presence of IL-2 (100 U/ml), IL-4 (5 U/ml), IL-6 (1000 U/ml), IL-7 (5 ng/ml), IL-12 (10 ng/ml), phytohemagglutinin (PHA; 250 ng/ml; HA-16, Murex, UK) and irradiated allogeneic EBV-B cells (LG2-EBV) as feeder cells (3×10^4 cells/well). To prepare the stimulator cells, monocyte-derived immature dendritic cells were incubated overnight with 50 μg/ml of peptide EVDPANSTY in the presence of β2-microglobulin (2.5 μg/ml), IL-4 (200 U/ml), GM-CSF (70 ng/ml) and TNFα (?1 ng/ml) and washed. After 3–5 weeks of proliferation, the CTL clones were re-stimulated every 1–2 weeks, in

2 ml of medium (5×10^5 CTL/well) with 2×10^5 irradiated LB34-MEL stimulator cells pulsed for 1 h with 1 μg/ml of peptide in the presence of β2-microglobulin, 1.5×10^6 feeder cells, IL-2, IL-4, and IL-7.

Dendritic cells

Peripheral blood was obtained from hemochromatosis patient LB672 as standard buffy coat preparations, which were laid down on a 15-ml of Lymphoprep layer (Axis-Shield PoCAS, Oslo, Norway) in 50-ml tubes. To minimize contamination of the PBMCs by platelets, the tubes were first centrifuged at 930 g for 20 min at room temperature. After removal of the top 20–25 ml, containing most of the platelets, the tubes were centrifuged at 430 g for 8 min at room temperature. The interphase containing the PBMCs was harvested and washed three times (or more) in cold phosphate buffer solution with 2 mM of EDTA in order to eliminate the remaining platelets. To generate autologous dendritic cells, PBMCs were left to adhere for 1 h at 37°C in culture flasks (FALCON, Becton Dickinson) at a density of 2×10^6 cells per cm^2 in RPMI 1640 supplemented with Hepes (2.38 g/l), AAG, antibiotics, and 10% FCS (hereafter referred to as complete RPMI medium). Nonadherent cells were discarded and adherent cells were cultured in the presence of IL-4 (200 U/ml) and GM-CSF (70 ng/ml) in complete RPMI medium. Cultures were fed on days 2 and 4 by removing one third of the volume and adding fresh medium with cytokines. They were frozen on day 5.

Transfection of COS cells and recognition assay based on TNF production

COS-7 cells (1.5×10^4) were distributed in flat-bottom microwells and cotransfected using 1 μl of Lipofectamine (Life Technologies, Merelbeke, Belgium) with pcDNAI/Amp (50 ng) containing either a *MAGE-4a* cDNA or a HLA-A1 cDNA. Transfected cells were incubated for 24 h at 37°C and 8% CO_2. The transfectants were then tested for their ability to stimulate the production of TNF by the CTL clone. Three thousand CTLs were added in the microwells containing either the COS-7 transfectants or tumor cells, in a total volume of 150 μl of complete IMDM supplemented with 25–50 U/ml of IL-2. After 24 h, the supernatant was collected and its TNF content was determined by testing its cytotoxic effect on WEHI-164 clone 13 cells in a MTT colorimetric assay (17–19).

Expression of *MAGE-4a* and *MAGE-4b*

Total cellular RNA from tumor tissues and cell lines was extracted by the guanidine-isothiocyanate/cesium chloride procedure (20). Reverse transcription was performed on 2 μg of total RNA in the presence of 200 units of M-MLV reverse transcriptase mixed with 4 μl of 5X First Strand Buffer (Life Technologies), 2 μl of 20 mM oligo-$(dT)_{15}$, 20 units of RNasin (Promega, Madison, WI), 2 μl of 100 mM of DTT (Life Technologies), 1 μl of each dNTP at 10 mM of each (Takara, Shiga, Japan), and DEPC-treated water, in a total volume of 20 μl. This mixture was incubated at 42°C for 90 min, then diluted to 100 μl with water. PCR amplification was performed on 2.5 μl (1/40th) of the reverse-transcribed RNA solution, in the presence of 0.625 units of Taq DNA polymerase in 1X PCR buffer (Takara), each primer at 0.4 μM, and each dNTP at 100 μM (Takara), in a total volume of 25 μl. RNA integrity was checked by reverse transcription and amplification of the β-actin mRNA. Sense primer was 5′-GAG-CAGACAGGCCAACCG-3′ (nucleotides −132 to −115 of the coding sequence of *MAGE-4a* and *MAGE-4b*) and antisense primer 5′-TCGCCCTCCATTGCAATTG-3′ (nucleotides 629–643 of the coding sequence of *MAGE-4a* and *MAGE-4b*). Amplifications were performed as follows: after a first denaturation step at 94°C for 4 min, 30 cycles of amplification were performed (1 min at 94°C, 1 min at 65°C, 2 min at 72°C), then a final extension step terminated the reaction. The PCR products of 775 bp were analyzed by agarose gel electrophoresis directly and after digestion by TaqI (Fermentas, Vilnius, Lithuania; restriction site at nucleotides 374–378 of the coding sequence of *MAGE-4a* and *MAGE-4b*) or SmuI (Life Technologies; restriction site at nucleotides 513–523 of the coding sequence of *MAGE-4a*).

Cytotoxicity assay

The EBV-B cells and tumor cells were labeled with 100 μCi of Na $(^{51}Cr)O$ for 1 h, washed and pulsed, if indicated, for 15 min with peptide. The CTLs were then added and chromium release was measured after incubation at 37°C for 4 h.

Results and discussion

MAGE-1 peptide EADPTGHSY and *MAGE-3* peptide EVDPIGHLY are presented to CTLs by HLA-A1. They are currently used in therapeutic vaccination trials (7, 21, 22). The homologous *MAGE-4a* peptide EVDPASNTY, which corresponds to the $MAGE\text{-}4a_{169-177}$ protein sequence, contains potential anchor residues for HLA-A1, namely D in position 3 and Y at the carboxy-terminus (23, 24). The residue at position 5 of this peptide is the unique difference between the *MAGE-4a* and *MAGE-4b* putative proteins. *MAGE-4b* codes for peptide EVDPTSNTY.

HLA-A1 molecules produced in *E. coli* were folded with the *MAGE-4a* peptide, demonstrating that the peptide binds efficiently to HLA-A1. The HLA-peptide complexes were biotinylated, and

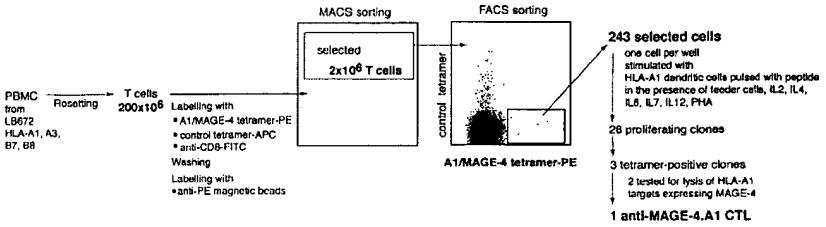

Fig. 1. **Overview of the procedure to obtain anti-*MAGE-4*.A1 cytolytic T lymphocyte (CTL) clones.** The control tetramer is a HLA-A1 tetramer containing an influenza peptide. MACS: magnetic cell sorting; FACS: fluorescence activated cell sorting. The cells represented on the FACS figure are CD8 T cells.

multimerized with avidin conjugated to phycoerythrin (PE). These multimers will be referred to as A1/*MAGE-4a* tetramers.

Isolation of CD8 T-cell clones labelled with tetramers

Two-hundred million T cells obtained from an individual without cancer were incubated with anti-CD8 antibodies, A1/*MAGE-4a* tetramers conjugated to PE, and control A1 tetramers folded with an influenza peptide and conjugated to allophycocyanin (APC) (Fig. 1). The cells were then incubated with an anti-PE antibody coupled to magnetic beads. Cells coated with beads were enriched by magnetic sorting. Approximately 2×10^6 cells were recovered. These cells were then introduced in a fluorescence-activated cell sorter, which selected the cells labelled with the A1/*MAGE-4a* tetramers conjugated to PE and cloned them at 1 cell per well. From the 243 microwells, 28 proliferating clones were obtained. Three of them were specifically labelled by A1/*MAGE-4a* tetramers. Only two clones, LB-709/D10 (clone D10) and LB-709/B1 (clone B1), proliferated enough to permit a functional analysis (Fig. 2).

Lysis of cells loaded with the *MAGE-4a* peptide

Clone D10, but not clone B1, had anti-MAGE*4a* lytic activity (Fig. 3). Titration of peptide EVDPASNTY revealed that half-maximal lysis of HLA-A1 target cells was obtained at a peptide concentration of 200 nM (Fig. 4). This is higher than for the previously identified *MAGE* antigenic peptides, for which values ranging from 0.05 to 100 nM were observed (22, 25–28). The *MAGE-4b* peptide, EVDPTSNTY, was not recognized by clone D10 (data not shown). The observation that lymphocytes, such as clone B1, can be labelled by a given peptide/MHC tetramer but fail to recognize peptide-pulsed cells have been described previously (29). This is probably an effect of the multimeric nature of the tetramers, which permits stable binding despite low affinity.

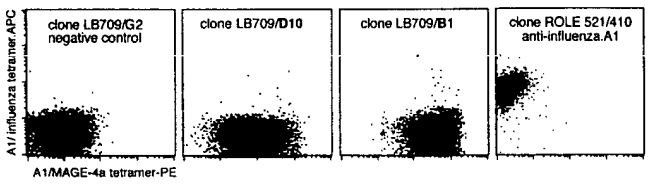

Fig. 2. **Labelling of T-cell clones with A1/*MAGE-4a* tetramers.** Cells were labelled for 15 min at 4°C with the A1-*MAGE-4a* tetramer (60 nM) conjugated to phycoerythrin (PE) and the control tetramer (20 nM) conjugated to allophycocyanin (APC). Anti-CD8 antibodies were then added for 15 min. The control HLA-A1 tetramer contains an influenza peptide, and specific staining with this tetramer was shown for anti-influenza A1 CTL clone ROLE 521/A10.

Recognition of cells expressing *MAGE-4a*

COS-7 cells cotransfected with a *MAGE-4* and *HLA-A1* cDNA constructs stimulated clone D10 to produce TNF, indicating that the *MAGE-4* antigen could be processed in these cells (Fig. 5). To identify the tumor cells expressing *MAGE-4a*, a fragment of 775 nucleotides that includes the peptide-coding sequence was amplified by PCR and digested by restriction enzymes TaqI and SmuI (Fig. 6). Only the *MAGE-4a* products can be digested by SmuI whereas TaqI can digest PCR products obtained from the two alleles. Sixteen out of

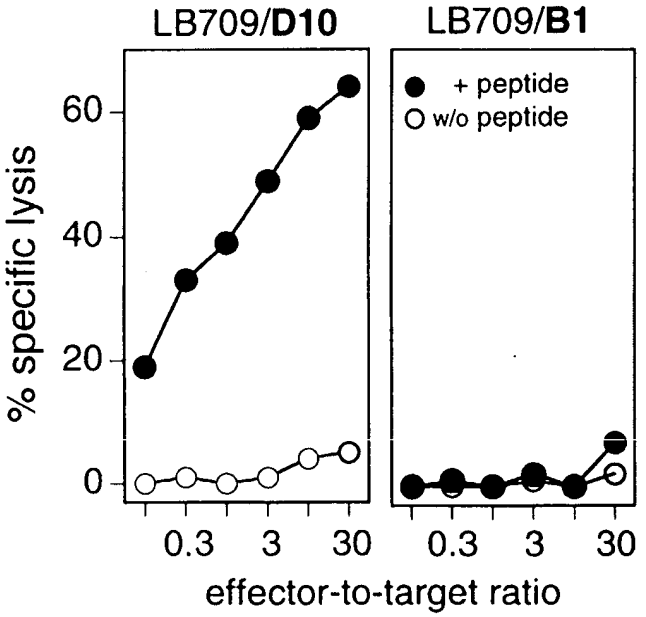

Fig. 3. **Lysis of HLA-A1 targets loaded with *MAGE-4a* peptide EVDPASNTY.** HLA-A1 BM21-Epstein-Barr virus-transformed B cells were ^{51}Cr-labelled for 1 h, incubated with 1 μg/ml of peptide for 15 min and incubated with the cytolytic T lymphocytes (CTLs) at indicated effector-to-target ratios. Chromium release was measured after 4 h.

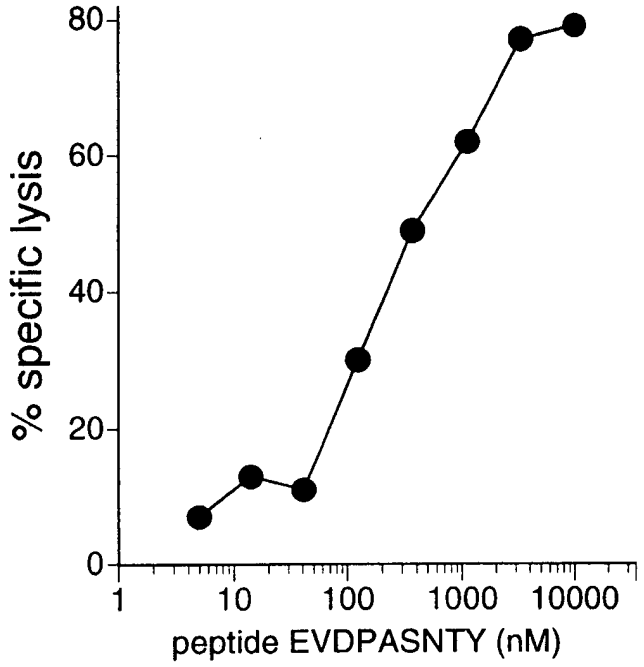

Fig. 4. **Titration of the *MAGE-4a* peptide.** BM21-Epstein-Barr virus-transformed B cells were ^{51}Cr-labelled and incubated for 15 min with threefold dilutions of the synthetic peptide. Cytolytic T lymphocyte D10 was subsequently added at an effector-to-target ratio of 5:1. Chromium release was measured 4 h later. The concentrations indicated in the figure correspond to the concentrations during the 4-h incubation.

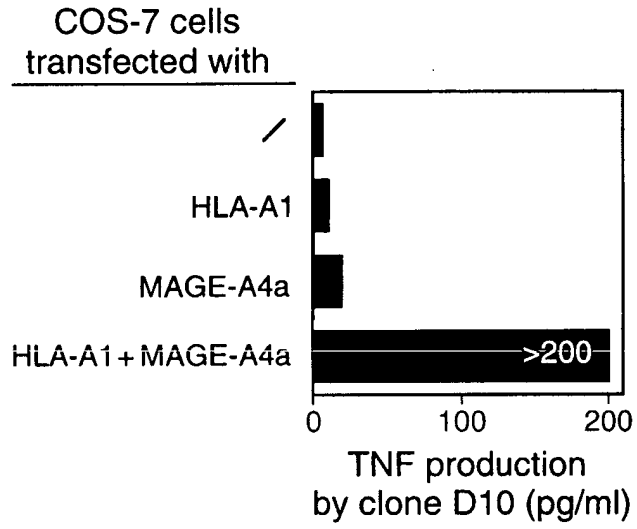

Fig. 5. **Recognition of cells transfected with *MAGE-4a*.** COS-7 cells were transiently transfected with a *MAGE-4a* and a HLA-A1 coding sequence, both inserted in expression vector pcDNAI/Amp. Transfections were performed with 15,000 COS-7 cells, 50 ng of each cDNA and 1 µl of Lipofectamine. One day after transfection, 3000 D10 cytolytic T lymphocytes were added to the transfected cells. TNF production was measured after overnight coculture by testing the toxicity of the supernatants for TNF-sensitive WEHI 164 clone 13 cells.

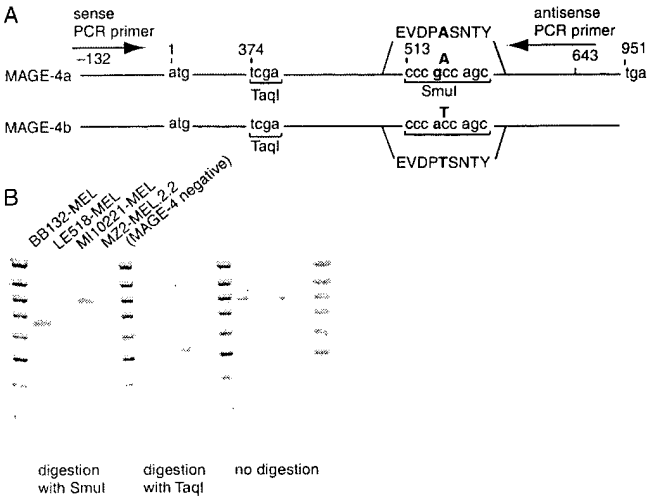

Fig. 6. **One-restriction enzyme can distinguish *MAGE-4a* from *MAGE-4b*.** (A) Nucleotides are numbered starting from the start codon. Restriction enzyme Smu 1, also named Fau 1, digests only *MAGE-4a* products, whereas restriction enzyme Taq 1 digests both *MAGE-4a* and *MAGE-4b* products. (B) Digestion of the PCR products. RNA was extracted from *MAGE-4*-expressing cell lines BB132-MEL, LE518A1-MEL and M10221-MEL, which were used as targets in Fig. 7. RNA was also extracted from cell line MZ2-MEL, which do not express *MAGE-4*. RT-PCR was performed according to the protocol described in (A). PCR products were analyzed by agarose gel electrophoresis after digestion by Smu 1, and Taq 1, or without digestion.

the 46 tumor samples (35%) that were previously found to be positive for the expression of *MAGE-4* were found to express *MAGE-4a*. Eleven out of 37 *MAGE-4*-positive tumor cell lines (30%) expressed the *MAGE-4a* allele. Two of these tumor cell lines were derived from

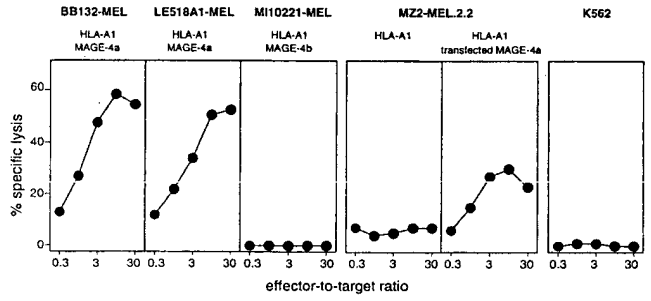

Fig. 7. **Lysis of HLA-A1 tumor cell lines expressing *MAGE-4a*.** Melanoma cell lines LE518A1 and BB132 express the *MAGE-4a* and *HLA-A1* genes. Melanoma cell line Mi10221 expresses the *MAGE-4b* and *HLA-A1* genes. The MZ2-MEL.2.2.*MAGE-4* cell line was obtained by stable transfection of the *MAGE-4a* coding sequence of HLA-A1 melanoma cell line MZ2-MEL.2.2. Cell line K562 is a target for natural killer cells. Target cells were ^{51}Cr-labelled and, if indicated, pulsed for 5 min with 1 µg/ml of peptide EVDPASNTY. They were then incubated for 4 h with cytolytic T lymphocyte clone D10 at the indicated effector-to-target ratios. Chromium release was measured after 4 h.

a HLA-A1 patient. They were lyzed by clone D10 whereas a HLA-A1 melanoma cell line expressing *MAGE-4b* was not lyzed (Fig. 7). A HLA-A1 tumor cell line, MZ2-MEL.2.2, transfected with a *MAGE-4a* coding sequence was also lyzed.

The new *MAGE-4a* antigenic peptide described here is presented by HLA-A1 molecules, which are expressed in the different major ethnic groups: Black (9%), Caucasoid (26%), Oriental (7%), and Amerindian (11%) (30). The identification of a large number of antigenic peptides presented by HLA class I and class II is likely to be important for the future of clinical trials with defined antigenic peptides. A large set of peptide/HLA combinations will widen the set of eligible patients. It will also facilitate the design of concurrent immunizations against several antigens. Such immunizations could increase the primary antitumor efficacy of the vaccine and decrease the risk of tumor escape by loss of antigen expression. Identification of additional *MAGE-4* antigenic peptides will be important because a number of tumors express *MAGE-4* without expressing *MAGE-A1* and *MAGE-A3*. This is the case for carcinomas of the lung (15%), head and neck (14%), esophagus (11%), and bladder (10%).

References

1. De Plaen E, Arden K, Traversari C et al. Structure, chromosomal localization and expression of twelve genes of the MAGE family. *Immunogenetics* 1994: **40**: 360–9.
2. Lurquin C, De Smet C, Brasseur F et al. Two members of the human *MAGEB* gene family located in Xp.21.3 are expressed in tumors of various histological origins. *Genomics* 1997: **46**: 397–408.
3. Lucas S, De Smet C, Arden KC et al. Identification of a new MAGE gene with tumor-specific expression by representational difference analysis. *Cancer Res* 1998: **58**: 743–52.
4. Chomez P, De Backer O, Bertrand M, De Plaen E, Boon T, Lucas S. An overview of the MAGE gene family with the identification of all human members of the family. *Cancer Res* 2001: **61**: 5544–51.
5. Haas GG Jr, D'Cruz OJ, De Bault LE. Distribution of human leukocyte antigen-ABC and -D/DR antigens in the unfixed human testis. *Am J Reprod Immunol Microbiol* 1988: **18**: 47–51.
6. Jäger E, Jäger D, Knuth A. Clinical cancer vaccine trials. *Curr Opin Immunol* 2002: **14**: 178–82.
7. Marchand M, van Baren N, Weynants P et al. Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene *MAGE-3* and presented by HLA-A1. *Int J Cancer* 1999: **80**: 219–30.
8. Thurner B, Haendle I, Roder C et al. Vaccination with MAGE-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. *J Exp Med* 1999: **190**: 1669–78.
9. Schuler-Thurner B, Schultz ES, Berger TG et al. Rapid induction of tumor-specific type I T helper cells in metastatic melanoma patients by vaccination with mature, cryopreserved, peptide-loaded monocyte-derived dendritic cells. *J Exp Med* 2002: **195**: 1279–88.
10. Schuler-Thurner B, Dieckmann D, Keikavoussi P et al. Mage-3 and influenza-matrix peptide-specific cytotoxic T cells are inducible in terminal stage HLA-A2.1$^+$ melanoma patients by mature monocyte-derived dendritic cells. *J Immunol* 2000: **165**: 3492–6.
11. Marchand M, Punt CJA, Aamdal S et al. Immunization of metastatic cancer patients with MAGE-3 protein combined with adjuvant SBAS-2: clinical report. *Eur J Cancer* 2003: **39**: 70–7.
12. De Plaen E, Naerhuyzen B, De Smet C, Szikora J-P, Boon T. Alternative promoters of gene MAGE4a. *Genomics* 1997: **40**: 305–13.
13. Duffour M-T, Chaux P, Lurquin C, Cornelis G, Boon T, van der Bruggen P. A MAGE-A4 peptide presented by HLA-A2 is recognized by cytolytic T lymphocytes. *Eur J Immunol* 1999: **29**: 3329–37.
14. Zhang Y, Stroobant V, Russo V, Boon T, van der Bruggen P. A MAGE-A4 peptide presented by HLA-B37 is recognized on human tumors by cytolytic T lymphocytes. *Tissue Antigens* 2002: **60**: 365–71.
15. Van Snick J, Vink A, Cayphas S, Uyttenhove C. Interleukin-HP1, a T cell-derived hybridoma growth factor that supports the in vitro growth of murine plasmocytomas. *J Exp Med* 1987: **165**: 641–9.
16. Altman JD, Moss PAH, Goulder PJR et al. Phenotypic analysis of antigen-specific T lymphocytes. *Science* 1996: **274**: 94–6.
17. Traversari C, van der Bruggen P, Van den Eynde B et al. Transfection and expression of a gene coding for a human melanoma antigen recognized by autologous cytolytic T lymphocytes. *Immunogenetics* 1992: **35**: 145–52.
18. Espevik T, Nissen-Meyer J. A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. *J Immunol Meth* 1986: **95**: 99–105.
19. Hansen MB, Nielsen SE, Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J Immunol Meth* 1989: **119**: 203–10.
20. Davis LG, Dibner MD, Battey JF. Guanidine isothiocyanate preparation of total RNA. In: Davis LG, Dibner MD, Battey JF, eds. Basic Methods in Molecular Biology. New York: Elsevier, 1986: 130–5.
21. Gaugler B, Van den Eynde B, van der Bruggen P et al. Human gene MAGE-3 codes for an antigen recognized on a melanoma by autologous cytolytic T lymphocytes. *J Exp Med* 1994: **179**: 921–30.
22. Traversari C, van der Bruggen P, Luescher IF et al. A nonapeptide encoded by human gene MAGE-1 is recognized on HLA-A1 by cytolytic T lymphocytes directed against tumor antigen MZ2-E. *J Exp Med* 1992: **176**: 1453–7.
23. DiBrino M, Parker KC, Shiloach J et al. Endogenous peptides with distinct amino acid anchor residue motifs bind to HLA-A1 and HLA-B8. *J Immunol* 1994: **152**: 620–31.
24. Kubo RT, Sette A, Grey HM et al. Definition of specific peptide motifs for four major HLA-A alleles. *J Immunol* 1994: **152**: 3913–24.

25. Chaux P, Luiten R, Demotte N et al. Identification of five MAGE-A1 epitopes recognized by cytolytic T lymphocytes obtained by *in vitro* stimulation with dendritic cells transduced with MAGE-A1. *J Immunol* 1999: **163**: 2928–36.
26. Schultz ES, Zhang Y, Knowles R et al. A MAGE-3 peptide recognized on HLA-B35 and HLA-A1 by cytolytic T lymphocytes. *Tissue Antigens* 2001: **57**: 103–9.
27. Luiten R, van der Bruggen P. A MAGE-A1 peptide is recognized on HLA-B7 human tumors by cytolytic T lymphocytes. *Tissue Antigens* 2000: **55**: 149–52.
28. van der Bruggen P, Szikora J-P, Boël P et al. Autologous cytolytic T lymphocytes recognize a MAGE-1 nonapeptide on melanomas expressing HLA-Cw*1601. *Eur J Immunol* 1994: **24**: 2134–40.
29. Dutoit V, Rubio-Godoy V, Pittet MJ et al. Degeneracy of antigen recognition as the molecular basis for the high frequency of naive A2/Melan-a peptide multimer$^+$ CD8$^+$ T cells in humans. *J Exp Med* 2002: **196**: 207–16.
30. Marsh SGE, Parham P, Barber LD. The HLA Factsbook. London: Academic Press, 2000.

A MAGE-A4 peptide presented by HLA-A2 is recognized by cytolytic T lymphocytes

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The *MAGE*-encoded antigens that are recognized by cytolytic T lymphocytes (CTL) are shared by many tumors and are strictly tumor specific. Clinical trials involving therapeutic vaccination of cancer patients with MAGE antigenic peptides or proteins are in progress. To increase the range of patients eligible for therapy with peptides, it is important to identify additional MAGE epitopes. We have used a method to identify CTL epitopes, which selects naturally processed peptides. $CD8^+$ T cells, obtained from individuals without cancer, were stimulated with autologous dendritic cells infected with a recombinant adenovirus containing the *MAGE-A4* coding sequence. Responder cell microcultures that specifically lysed autologous EBV-transformed B cells infected with vaccinia-MAGE-A4 were cloned using autologous stimulator cells infected with a *Yersinia enterocolitica* carrying the MAGE-A4 sequence. An anti-MAGE-A4 CTL clone was obtained and the epitope was found to be decapeptide GVYDGREHTV (amino acids 230–239) presented by HLA-A2 molecules. The CTL clone lysed HLA-A2 tumor cells expressing *MAGE-A4*. This is the first reported antigenic peptide encoded by *MAGE-A4*. It may be valuable for cancer immunotherapy because *MAGE-A4* is expressed in 51 % of lung carcinomas and 63 % of esophageal carcinomas, whereas about 50 % of Caucasians and Asians express HLA-A2.

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1 Introduction

CTL directed against human tumor cells have been obtained by cultivating irradiated tumor cells with autologous lymphocytes from cancer patients [1, 2]. Several of these CTL have been used as tools to isolate genes that code for the antigens recognized on the tumors, such as those of the *MAGE* gene family, which includes at least 17 related genes, namely *MAGE-1* to *12* (now named *MAGE-A1* to *A12*), *MAGE-B1* to *B4* and *MAGE-C1* [3–7]. Genes of this family are expressed in tumors of various histological types but not in normal tissues, except male germ-line cells, which do not present HLA class I molecules and are therefore unable to present antigens to CTL [8, 9]. Hence, antigens encoded by *MAGE* genes should be strictly tumor specific.

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Abbreviations: DC: Dendritic cells **IMDM:** Iscove's modified Dulbecco's medium **EBV-B:** Epstein-Barr virus-infected B cells **ALVAC-MAGE-A4:** Avian poxvirus ALVAC containing the MAGE-A4 coding sequence **MOI:** Multiplicity of infection

The gene *MAGE-A4* presents two alleles, *MAGE-A4a* and *MAGE-A4b*, which code for proteins differing by a single amino acid [4]. It contains eight alternative first exons that are spliced to unique second and third exons. The entire open reading frame is always located in the third exon [10]. The role of these different first exons, each with their own promoter, is unknown. *MAGE-A4* is not only expressed in male germ-line cells but also in the placenta, which does not present HLA class I molecules [11]. In addition, the placenta produces a tryptophan-catabolizing enzyme, which suppresses T cell activity [12]. *MAGE-A4* is frequently expressed in carcinomas of the esophagus (63 %), the head and neck (53 %), the lung (51 %) and the bladder (33 %), and also in metastatic melanomas (28 %). When a tumor sample is found to be positive for *MAGE-A4*, this gene is usually expressed at a very high level. MAGE-A4-derived antigens may thus be valuable for cancer immunotherapy but no such antigen has been identified so far.

Several strategies can be considered to find CTL epitopes encoded by *MAGE-A4*. A first approach requires the location of candidate peptides carrying consensus

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anchor motives for a certain HLA in the sequence of the MAGE-A4 protein. These peptides are then tested for HLA binding and the best binders are loaded on APC to stimulate T lymphocytes *in vitro* [13, 14]. Epitopes encoded by *MAGE-A1, A2, A3,* and *B2* have been identified by this approach [14–17]. Although straightforward, the major drawback of this approach is that many peptide-specific CTL do not recognize HLA-matched tumor cells expressing the protein endogenously [18, 19]. We followed a second approach that ought to lead to epitopes that are well processed. Here, dendritic cells (DC) were modified to express *MAGE-A4* and used to stimulate autologous $CD8^+$ T lymphocytes *in vitro*. DC are potent APC and they can activate naive T lymphocytes [20]. There is so far no effective method to transfect human DC. However, viral delivery vectors have been used successfully: it has been shown that DC can be infected with recombinant adenoviruses and poxviruses, and that infection with a recombinant adenovirus leads to a high expression of the transgene, which remains elevated for more than 1 week [21, 22].

We report here the identification of a MAGE-A4 antigenic peptide presented to CTL by HLA-A2 molecules, using an approach based on DC infected with an adenoviral vector encoding *MAGE-A4*.

2 Results

2.1 Activation of a CTL specific for a MAGE-A4 epitope

The experiments were performed with blood cells from an individual without cancer, hemochromatosis patient LB1137. DC were obtained by culturing blood monocytes for 4–6 days in medium supplemented with GM-CSF and IL-4. Cells prepared by this procedure have been referred to as "immature DC" [23, 24]. DC were infected with an adenovirus engineered to contain the *MAGE-A4* sequence under the influence of a CMV promoter (adeno-MAGE-A4). Infected DC were distributed in microwells to stimulate autologous $CD8^+$ T cells, once per week for 3 weeks. Since one of the essential features of a mature DC is to produce IL-12, the medium was supplemented with IL-12. Recently, we have also used mature stimulator DC, obtained by treating immature DC for 2 days with TNFα, IL1-β, IL-6, and PGE_2 [25]. Very high proliferation of T cells was observed in all the microcultures, but we did not obtain effectors with anti-MAGE lytic activity. We have tentatively concluded that the anti-MAGE CTL were overwhelmed by other T cells that proliferated very rapidly after stimulation by mature DC and, therefore; we continue to use immature stimulator DC in medium supplemented with IL-12.

Responder cells of each microculture were tested on day 21 for their lytic activity on autologous EBV-transformed B (EBV-B) cells infected with a vaccinia virus encoding *MAGE-A4* (vaccinia-MAGE-A4). The reason for using vaccinia-MAGE-A4 instead of adeno-MAGE-A4 was primarily that adenoviral vectors rarely infect EBV-B cells. Moreover, vaccinia-infected targets are not lysed by CTL that would have been activated by antigens derived from the adenoviral vector.

A microculture displaying anti-MAGE-A4 reactivity was cloned by limiting dilution in the presence of autologous EBV-B stimulator cells, IL-2 and allogeneic EBV-B feeder cells. EBV-B stimulators were used for the cloning step because of the limited supply of autologous DC. To stimulate only the anti-MAGE-A4 CTL clones, while avoiding the proliferation of CTL against the adenoviral vector, we infected the irradiated EBV-B stimulators with recombinant *Yersinia enterocolitica* bacteria producing MAGE-A4 (Yersinia-MAGE-A4). These recombinant bacteria expressed a YopE-MAGE-A4 fusion gene allowing the translocation of a MAGE-A4 fusion protein into the cytosol of mammalian cells (see Sect. 4.3). We had previously verified that HLA-A1 EBV-B cells infected with Yersinia-MAGE-A1 were capable of stimulating the release of IFN-γ by CTL clone 82/30 [3], which is directed against a MAGE-A1 epitope presented by HLA-A1 (data not shown). After a few weekly restimulations, the growing CTL clones were tested for their lytic activity. Several clones were obtained which lysed autologous EBV-B cells only when infected with vaccinia-MAGE-A4. Results are shown for representative CTL clone H4/13, hereafter referred to as CTL clone 13 (Fig. 1).

We compared DC infected with adeno-MAGE-A4 or with a canarypox virus containing the coding sequence of

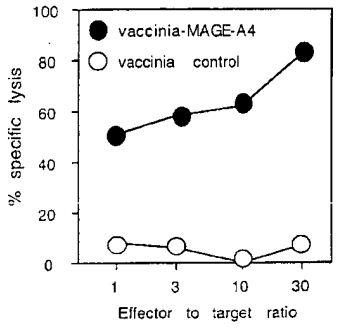

Figure 1. Lysis by CTL clone 13 of autologous EBV-B cells infected with vaccinia-MAGE-A4. EBV-B cells were infected for 2 h with the vaccinia vector, ^{51}Cr-labeled for 1 h and incubated with the CTL at the indicated E/T ratios. Chromium release was measured after 4 h.

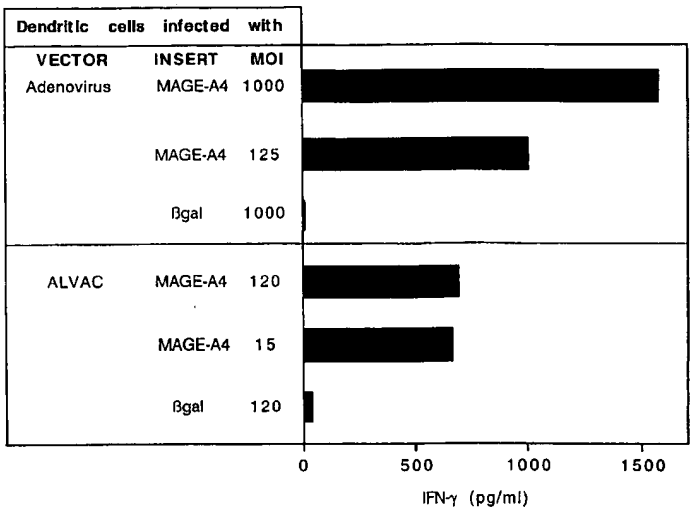

Figure 2. Recognition of DC infected with the viral constructs expressing *MAGE-A4*. HLA-A2 DC were infected with adeno-MAGE-A4, ALVAC-MAGE-A4, or control vectors containing the β-galactosidase (βgal) reporter gene, at the indicated MOI. When DC were infected with an adenoviral vector at a MOI of 1 000, the level of infection was usually higher than 80 % as assessed with a β-galactosidase reporter. The plateau of infection was obtained for ALVAC at a MOI of 15, which usually gives 30–40 % infected cells. Twenty-four hours after infection, 2 500 CTL 13 were added and the concentration of IFN-γ in the supernatant was measured after 24 h.

MAGE-A4 (ALVAC-MAGE-A4) for their ability to stimulate CTL clone 13. DC infected with adeno-MAGE-A4 stimulated the secretion of a high amount of IFN-γ by CTL 13, whereas cells infected with ALVAC-MAGE-A4 were less efficient stimulators (Fig. 2). In a few other experiments, DC infected with ALVAC-MAGE-A4 were used to stimulate anti-MAGE-A4 CTL precursors and microcultures displaying anti-MAGE-A4 reactivity were obtained (data not shown).

2.2 A MAGE-A4 peptide presented by HLA-A2

Patient LB1137 was typed HLA-A2, A3, B4402, B60, Cw3, Cw5. The recognition of autologous EBV-B cells infected with vaccinia-MAGE-A4 by CTL clone 13 was inhibited in the presence of anti-HLA-A,B,C and anti-HLA-A2 mAb, demonstrating that the MAGE-A4 epitope was presented by HLA-A2 molecules (data not shown). To confirm that the epitope recognized by this CTL was encoded by *MAGE-A4*, COS-7 cells were cotransfected with the *MAGE-A4* cDNA and the HLA-A2 gene. These transfectants stimulated CTL clone 13 to produce TNF (Fig. 3).

To identify the region of *MAGE-A4* that codes for the peptide recognized by clone 13, we constructed truncated coding sequences of *MAGE-A4* of various lengths. PCR reactions were performed using a *MAGE-A4* cDNA as the template, an upstream primer consisting of the first nucleotides of the open reading frame of *MAGE-A4*, and eight downstream primers. This led to the amplification of eight fragments of *MAGE-A4*, the longer one con-

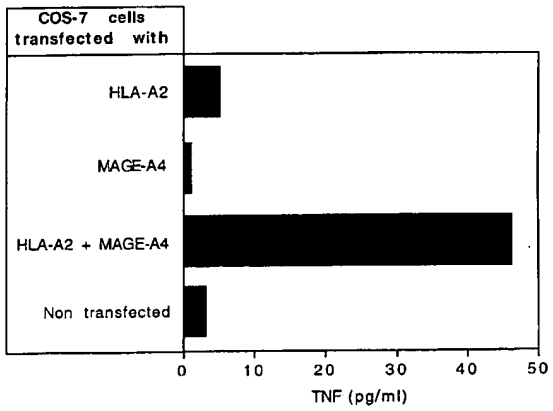

Figure 3. Recognition by CTL clone 13 of a MAGE-A4 antigen presented by HLA-A2. COS-7 cells were transfected with expression vectors containing the HLA-A2 gene and the *MAGE-A4* cDNA. CTL 13 cells (2000) were added to the transfectants and the TNF content of the supernatant was estimated after 24 h by testing its toxicity on WEHI-164 clone 13 cells.

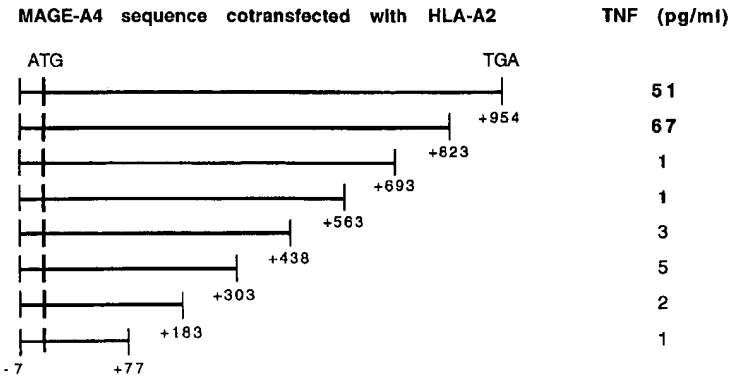

Figure 4. Identification of a *MAGE-A4* fragment coding for the antigen recognized by CTL clone 13. PCR reactions were performed on the *MAGE-A4* cDNA to obtain eight truncated *MAGE-A4* cDNA that were cloned in an expression vector. Nucleotides were numbered, starting from the first nucleotide of the open reading frame. HeLa cells (20 000) were cotransfected with expression vectors containing the HLA-A2 gene and the *MAGE-A4* fragments. CTL 13 cells (2000) were added 24 h later to the transfectants and the TNF concentration of the supernatant was measured 20 h later, by testing its toxicity on WEHI-164 clone 13 cells.

taining the entire open reading frame of *MAGE-A4*. These fragments were inserted into an expression vector and transfected into HeLa cells, along with the HLA-A2 gene, to test their ability to code for the antigen. Transfection with the two longer inserts stimulated the secretion of TNF by CTL 13, while the other constructs were negative (Fig. 4). This indicated that the C terminus of the peptide had to be encoded by nucleotides 693 to 823 of the open reading frame. The sequence of the MAGE-A4 protein encoded by this region was screened for the presence of a peptide carrying consensus anchor residues for HLA-A2 (L or V in position 2 and I, L, V, or M in position 9 or 10) [26]. We found only one peptide of ten amino acids, GVYDGREHTV (amino acids 230–239), that satisfied this criterion. Peptide GVYDGREHTV was found to sensitize autologous target cells to lysis by clone 13, while nonapeptide VYDGREHTV did not. Half-maximal lysis was obtained at a peptide concentration of 30 nM (Fig. 5). This result is in the same range as for other MAGE peptides: 5 nM for the MAGE-A1 peptide presented by HLA-A1, and 25 nM for the MAGE-A1 peptide presented by HLA-Cw*1601 [27, 28].

2.3 Recognition of HLA-A2 tumor cell lines expressing MAGE-A4

Since we used DC expressing *MAGE-A4* to activate CTL clone 13, it was important to verify that tumor cells also efficiently processed the MAGE-A4 antigen. Three HLA-A2 melanoma and a sarcoma cell line expressing *MAGE-A4* were tested and all were found to be lysed by CTL 13 (Fig. 6).

3 Discussion

The procedure described here appears to be efficient for the activation of anti-MAGE CTL precursors and does not require blood cells from cancer patients. We have observed better survival and stimulation capacities for DC infected with an adenoviral vector as compared with poxvirus-infected DC. However, we cannot conclude from our few experiments that adenovirus-infected DC are better stimulators for activation of anti-MAGE CTL precursors from individuals without cancer. A potential

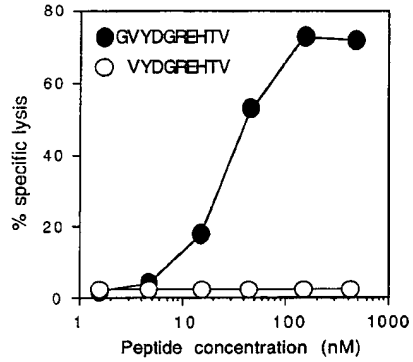

Figure 5. Lysis by CTL clone 13 of autologous EBV-B cells incubated with peptide GVYDGREHTV. EBV-B cells were ^{51}Cr-labeled for 1 h and incubated with CTL 13 at an E/T ratio of 10:1 in the presence of the MAGE-A4 peptide at the concentrations indicated. Chromium release was measured after 4 h.

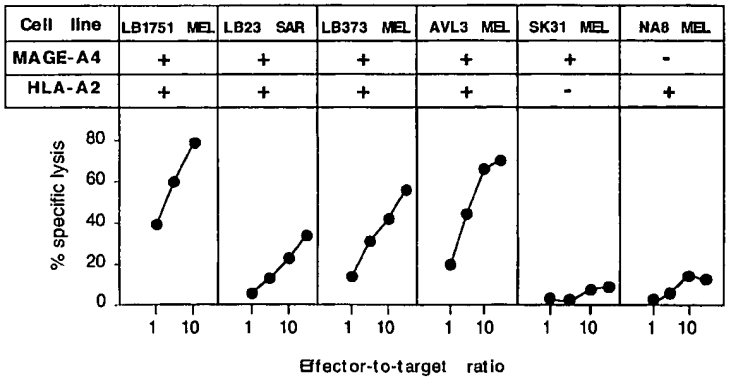

Figure 6. Lysis by CTL clone 13 of HLA-A2 tumor cell lines expressing MAGE-A4. Target cells were ^{51}Cr-labeled for 1 h and incubated with CTL at various E/T ratios. Chromium release was measured after 4 h. MEL stands for melanoma and SAR for sarcoma.

drawback of our approach, which is based on the use of a recombinant virus for antigen delivery, is that CTL precursors directed against viral epitopes can easily be activated. We attempted to circumvent this problem by using different vectors for the stimulation of the microcultures, for testing the lytic activity with the responder T cells, and for the cloning step.

Interestingly, the MAGE-A4-derived peptide, GVYDGREHTV, and the recently identified MAGE-A10 peptide, GLYDGMEHL, which is also presented by HLA-A2 but not recognized by CTL 13, are located in homologous regions of the protein [29]. This was also observed for the MAGE-A3 and MAGE-A1 antigens presented by HLA-A1 [27, 30]. This may suggest that some fragments are processed more efficiently than others. The identification of a series of additional antigens in these MAGE proteins may help to define rules for the processing of proteins into antigenic peptides.

After infection with viral vectors containing an entire *MAGE* coding sequence, only those antigenic peptides processed efficiently by the infected DC are able to activate anti-MAGE CTL. These peptides are most likely those that are also well processed and presented at the surface of MAGE$^+$ tumor cells. Accordingly, CTL clone 13 lysed all four HLA-A2 tumor cell lines expressing *MAGE-A4*. The MAGE-A4 epitope described here is also efficiently processed by DC and EBV-B cells infected with a viral vector coding for *MAGE-A4*. In contrast, the MAGE-$A3_{271-279}$ epitope presented by HLA-A2, which was identified by the peptide approach [15], appeared to be poorly processed: EBV-B cells infected with vaccinia-MAGE-A3 were not lysed by CTL clone 297/22 (data not shown). This is probably due to the inaccurate cleavage of the MAGE-A3 protein during its processing [31]. Even though some tumors that appeared to express a high level of *MAGE-A3* showed significant lysis by CTL clone 297/22 and by other CTL clones directed against the same peptide [15, 32, 33], we feel that the MAGE-A4 epitope presented here could be a better candidate for immunotherapy, because it is processed more efficiently.

MAGE-A4 is expressed in a significant proportion of tumors, and 49 % of Caucasians and Asians express the HLA-A2 allele. This MAGE-A4 epitope could be particularly interesting for those patients whose tumor expresses *MAGE-A4* but not *MAGE-A1* or *MAGE-A3*, which is the case in a number of carcinomas of the lung (15 %), the head and neck (14 %), the esophagus (11 %), and the bladder (10 %). Different modes of immunization can be envisaged: a synthetic peptide, DC pulsed with this peptide, a MAGE-A4 protein, or viral vectors encoding the epitope. In clinical trials, the availability of monoclonal and polyclonal anti-MAGE-A4 antibodies may facilitate the detection of the MAGE-A4 protein, both in tissue sections and in the serum of cancer patients [34, 35].

In future trials, it will be essential to have reliable monitoring of the CTL response against the immunizing antigen. One possibility, which unfortunately narrows the analysis to certain epitopes, is the use of relevant peptides to label T cell receptors directly with soluble HLA tetramers presenting the relevant peptide [36]. HLA-A2 tetramers folded with melanoma peptides have been shown to detect anti-Melan-A and anti-tyrosinase CTL in melanoma-infiltrated lymph nodes [37]. This approach requires prior identification of these antigenic peptides. An HLA-A2 tetramer folded with this MAGE-A4 peptide can now be constructed, tested with our CTL and then used for monitoring.

4 Materials and methods

4.1 Cell lines and peptides

Melanoma cell lines LB1751-MEL, AVL3-MEL, LB373-MEL.4.0, LB23-SAR clone 2, SK31-MEL and NA8-MEL were grown in Iscove's modified Dulbecco's medium (IMDM) containing 10 % FCS and supplemented with 0.24 mM L-asparagine, 0.55 mM L-arginine and 1.5 mM L-glutamine (AAG); for LB1751-MEL and AVL3-MEL the medium was also supplemented with Hites [38]. COS-7 cells and HeLa cells were maintained in DMEM with 10 % FCS. WEHI-164 clone 13 cells were cultured in RPMI with 5 % FCS. Culture media were purchased from Gibco-BRL (Merelbeke, Belgium). Peptide were synthesized on solid phase using Fmoc for transient N-terminal protection and were characterized using mass spectrometry. All peptides were > 80 % pure, as indicated by analytical HPLC. Lyophilized peptides were dissolved in DMSO and stored at – 20 °C.

4.2 Poxviruses and adenoviruses

Recombinant canarypoxvirus ALVAC-MAGE-A4 (vCP1544) and ALVAC-βgal (vCP325) and the vaccinia viruses encoding MAGE-A4 (vP1545) were produced by Virogenetics (Troy, NY). The plasmid pAd-CMVIcpA-MAGE-A4 (containing the MAGE-A4 cDNA under the control of the CMV promoter) was obtained by inserting the complete *MAGE-A4* cDNA into the NotI site of vector pAd-CMVIcpA (kindly provided by Celia Garcia-Martinez and Thierry Ragot, Institut Gustave Roussy, France). The recombinant adenovirus adeno-MAGE-A4 was constructed by *in vivo* homologous recombination in cell line 293 between pAd-CMVIcpA-MAGE-A4 and adeno-βgal genomic DNA [39]. The recombinant adenovirus was plaque purified, propagated in cell line 293 and purified by double cesium chloride density centrifugation.

4.3 Construction of a recombinant *Yersinia* and infection of EBV-B cell lines

The wild-type *Y. enterocolitica* is an extracellular bacterium causing gastro-intestinal syndromes in humans. *Y. enterocolitica* adheres to the surface of target cells and possesses a virulence apparatus, called the Yop virulon, which enables the translocation of toxic effector proteins, including YopE, into the cytosol of the host cell [40, 41]. A new strain, MRS40 (pABL403), has recently been constructed, in which the genes encoding toxic Yop proteins are mutated or truncated [42]. Interestingly, this polymutant strain maintains its ability to translocate proteins in fusion with a truncated YopE into the cytosol of eukaryotic cells, but does not elicit cytotoxicity and can therefore be used as a vector to inject a protein into the cytosol of eukaryotic cells. The sequence encoding protein MAGE-A4 was inserted in frame with a sequence encoding the first 130 amino acids of YopE, which was inserted into vector pMS621 [40]. Plasmid pMS621-MAGE-A4 was electroporated in *Escherichia coli* strain SM10 and was mobilized by SM10 into *Y. enterocolitica* MRS40 (pABL403). Recombinant MRS40 (pABL403) clones were selected on agar-containing medium, supplemented with 35 μg/ml nalidixic acid, 1 mM sodium m-arsenite and 12 μg/ml chloramphenicol [43].

One colony of *Y. enterocolitica* MRS40 (pABL403) containing pMS621-MAGE-A4 was grown overnight at 28 °C in LB medium supplemented with 35 μg/ml nalidixic acid, 1 mM sodium m-arsenite and 12 μg/ml chloramphenicol. This culture was diluted in order to obtain an OD of 0.2 at 600 nm and cultured at 28 °C for approximately 2 h. The bacteria were then washed in 0.9 % NaCl and resuspended at 10^8 per ml in 0.9 % NaCl, assuming that a culture giving an OD_{600} equal to 1 contains 5×10^8 bacteria per ml. Irradiated EBV-B cells were resuspended at 10^6 in 3.8 ml of RPMI without antibiotics, supplemented with 10 % FCS and AAG. Then, 200 μl of the bacterial suspension were added. Two hours after infection, 30 μg/ml gentamicin was added for the next 2 h. The cells were finally washed three times, before they were used as stimulator cells.

4.4 Responder T cells and APC

Peripheral blood was obtained from hemochromatosis patients as standard buffy coat preparations. PBMC were isolated by centrifugation on Lymphoprep (Nycomed Pharma, Oslo, Norway). The interphase containing the PBMC was harvested and washed three times in cold PBS with 2 mM EDTA to eliminate the remaining platelets. PBMC were depleted of T cells by rosetting with 2-aminoethylisothiouronium (Sigma, St Louis, MO)-treated SRBC. Rosetted T cells were treated with 160 mM NH_4Cl to lyse the SRBC, and washed. $CD8^+$ T lymphocytes were isolated from rosetted T cells by positive selection using an anti-CD8 mAb coupled to magnetic microbeads (Miltenyi Biotech, Germany). Sorted cells were frozen. The day before the first stimulation, $CD8^+$ T cells were thawed and cultured overnight in IMDM with 5 U/ml of IL-2, AAG and 10 % hman serum. The lymphocyte-depleted PBMC were left to adhere for 2 h. Nonadherent cells were discarded and adherent cells were cultured in the presence of 100 U/ml IL-4 and 100 ng/ml GM-CSF in complete medium. Cultures were fed on day 2 by adding fresh medium with IL-4 and GM-CSF. They were frozen on day 4 and thawed the day before each stimulation. EBV-B cells were obtained following the standard protocol.

4.5 Activation of anti-MAGE CTL precursors

Autologous DC from donor LB1137 were infected for 2 h with the adeno-MAGE-A4 construct at a multiplicity of infection (MOI) of 200, and washed. $CD8^+$ T lymphocytes (150 000) and 30 000 infected DC were cocultured in micro-

wells in 200 μl IMDM, AAG and 10 % human serum and supplemented with 1 000 U/ml IL-6 and 10 ng/ml IL-12. The $CD8^+$ lymphocytes were restimulated weekly with autologous DC freshly infected with adeno-MAGE-A4 and grown from day 7 in medium with 10 U/ml IL-2 and 5 ng/ml IL-7.

4.6 Cytotoxicity assay

Autologous EBV-B target cells were infected for 2 h with vaccinia constructs (MOI 20) before labeling for 1 h with $Na^{51}CrO_4$. They were added to the responder cells of each microculture at an E/T ratio of ~40:1. To test the microcultures, unlabeled K562 cells were added (5×10^4 per V-bottomed microwell) to block natural killer activity. Chromium release was measured after 4 h of incubation at 37 °C.

4.7 CTL clone 13: limiting dilution and culture conditions

A microculture containing cells with anti-MAGE-A4 lytic activity was cloned by limiting dilution using autologous EBV-B cells infected with a recombinant Yersinia-MAGE-A4 construct as stimulators, and allogeneic irradiated EBV-B cells (LG2-EBV) as feeder cells, in medium with 50 U/ml IL-2. CTL clone 13 was maintained in culture by weekly restimulation in 24-well plates with either EBV-B cells infected with Yersinia-MAGE-A4 (10^5) or 0.5 μg/ml PHA in complete IMDM with 50 U/ml IL-2 and 10^6 LG2-EBV feeder cells, as well as with 15 μg/ml gentamicin when Yersinia-infected cells were used as stimulator cells.

4.8 Construction of plasmids containing *MAGE-A4* minigenes

To construct truncated *MAGE-A4* cDNA, PCR reactions were performed using a *MAGE-A4* cDNA as the template, an upstream primer consisting of the first nucleotides of the open reading frame of *MAGE-A4* (position −7 to +20 in the *MAGE-A4* open reading frame, position 0 corresponding to the A of the start codon) and eight downstream primers, separated from each other by approximately 100–120 bp in the open reading frame of *MAGE-A4*. The PCR was performed for 30 cycles (1 min at 94 °C, 2 min at 63 °C and 3 min at 72 °C). PCR products were ligated into the pcDNA3.1/V5/His-TOPO vector (Topo TA cloning kit, Invitrogen).

4.9 Transfection of COS-7 and HeLa cells and TNF assay

Transfection experiments were performed using DMRIE-C for COS-7 cells and LipofectAMINE for HeLa cells (both from GIBCO, Merelbeke, Belgium). Briefly, 15 000 COS-7 cells or 20 000 HeLa cells were treated in one microwell with 50 ng plasmid pcDNAI/Amp containing the HLA-A2 genomic DNA, 50 ng plasmid containing the *MAGE-A4* cDNA (truncated or not) and 1 μl of DMRIE-C or LipofectAMINE. These transfectants were tested for antigen expression in a TNF assay after 24 h, as previously described [44]. Briefly, 2 000 CTL were added to the microwells containing the cells, in a total volume of 100 μl IMDM complete medium with 25 U/ml IL-2. After 24 h, the supernatant was collected and its TNF content was determined by testing its cytotoxic effect on cells of WEHI-164 clone 13 in a MTT colorimetric assay [44–46].

4.10 IFN-γ assay

CTL (2500) were added to 20 000 DC in microplates containing 100 μl IMDM, AAG, 10 % human serum, and 25 U/ml IL-2. After 24 h, the supernatant was collected and its IFN-γ content was determined by ELISA developed in our laboratory using reagents from Biosource (Fleurus, Belgium).

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5 References

1 **Hérin, M., Lemoine, C., Weynants, P., Vessière, F., Van Pel, A., Knuth, A., Devos, R. and Boon, T.,** Production of stable cytolytic T-cell clones directed against autologous human melanoma. *Int. J. Cancer* 1987. **39:** 390–396.

2 **Anichini, A., Fossati, G. and Parmiani, G.,** Clonal analysis of the cytolytic T-cell response to human tumors. *Immunol. Today* 1987. **8:** 385–389.

3 **van der Bruggen, P., Traversari, C., Chomez, P., Lurquin, C., De Plaen, E., Van den Eynde, B., Knuth, A. and Boon, T.,** A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 1991. **254:** 1643–1647.

4 **De Plaen, E., Arden, K., Traversari, C., Gaforio, J. J., Szikora, J.-P., De Smet, C., Brasseur, F., van der Bruggen, P., Lethé, B., Lurquin, C., Brasseur, R., Chomez, P., De Backer, O., Cavenee, W. and Boon, T.,** Structure, chromosomal localization and expression of twelve genes of the MAGE family. *Immunogenetics* 1994. **40:** 360–369.

5 **Lucas, S., De Smet, C., Arden, K. C., Viars, C. S., Lethé, B., Lurquin, C. and Boon, T.,** Identification of a new MAGE gene with tumor-specific expression by representational difference analysis. *Cancer Res.* 1998. **58:** 743–752.

6 **Lurquin, C., De Smet, C., Brasseur, F., Muscatelli, F., Martelange, V., De Plaen, E., Brasseur, R., Monaco, A. P. and Boon, T.,** Two members of the human *MAGEB* gene family located in Xp.21.3 are expressed in tumors of various histological origins. *Genomics* 1997. **46:** 397–408.

7 **Muscatelli, F., Walker, A. P., De Plaen, E., Stafford, A. N. and Monaco, A. P.,** Isolation and characterization of a new MAGE gene family in the Xp21.3 region. *Proc. Natl. Acad. Sci. USA* 1995. **92:** 4987–4991.

8 **Haas, G. G., Jr., D'Cruz, O. J. and De Bault, L. E.,** Distribution of human leukocyte antigen-ABC and -D/DR antigens in the unfixed human testis. *Am. J. Reprod. Immunol. Microbiol.* 1988. **18:** 47–51.

9 **Takahashi, K., Shichijo, S., Noguchi, M., Hirohata, M. and Itoh, K.,** Identification of MAGE-1 and MAGE-4 proteins in spermatogonia and primary spermatocytes of testis. *Cancer Res.* 1995. **55:** 3478–3482.

10 **De Plaen, E., Naerhuyzen, B., De Smet, C., Szikora, J.-P. and Boon, T.,** Alternative promoters of gene MAGE4a. *Genomics* 1997. **40:** 305–313.

11 **Hunt, J. S.,** Immunobiology of pregnancy. *Curr. Opin. Immunol.* 1992. **4:** 591–596.

12 **Munn, D. H., Zhou, M., Attwood, J. T., Bondarev, I., Conway, S. J., Marshall, B., Brown, C. and Mellor, A. L.,** Prevention of allogeneic fetal rejection by tryptophan catabolism. *Science* 1998. **281:** 1191–1193.

13 **Jung, s. and Schluesener, H. J.,** Human T lymphocytes recognize a peptide of single point-mutated, oncogenic ras proteins. *J. Exp. Med.* 1991. **173:** 273–276.

14 **Celis, E., Tsai, V., Crimi, C., DeMars, R., Wentworth, P. A., Chesnut, R. W., Grey, H. M., Sette, A. and Serra, H. M.,** Induction of anti-tumor cytotoxic T lymphocytes in normal humans using primary cultures and synthetic peptide epitopes. *Proc. Natl. Acad. Sci. USA* 1994. **91:** 2105–2109.

15 **van der Bruggen, P., Bastin, J., Gajewski, T., Coulie, P. G., Boël, P., De Smet, C., Traversari, C., Townsend, A. and Boon, T.,** A peptide encoded by human gene MAGE-3 and presented by HLA-A2 induces cytolytic T lymphocytes that recognize tumor cells expressing MAGE-3. *Eur. J. Immunol.* 1994. **24:** 3038–3043.

16 **Herman, J., van der Bruggen, P., Luescher, I., Mandruzzato, S., Romero, P., Thonnard, J., Fleischhauer, K., Boon, T. and Coulie, P. G.,** A peptide encoded by the human gene MAGE-3 and presented by HLA-B44 induces cytolytic T lymphocytes that recognize tumor cells expressing MAGE-3. *Immunogenetics* 1996. **43:** 377–383.

17 **Fleischhauer, K., Gattinoni, L., Dalerba, P., Lauvau, G., Zanaria, E., Dabovic, B., van Endert, P., Bordignon, C. and Traversari, C.,** The DAM gene family encodes a new group of tumor-specific antigens recognized by human leukocyte antigen A2-restricted cytotoxic T lymphocytes. *Cancer Res.* 1998. **58:** 2969–2972.

18 **Purcell, A. W., Chen, W., Ede, N. J., Gorman, J. J., Fecondo, J. V., Jackson, D. C., Zhao, Y. and McCluskey, J.,** Avoidance of self-reactivity results in skewed CTL responses to rare components of synthetic immunogens. *J. Immunol.* 1998. **160:** 1085–1090.

19 **Van den Eynde, B. and van der Bruggen, P.,** T cell-defined tumor antigens. *Curr. Opin. Immunol.* 1997. **9:** 684–693.

20 **Steinman, R.,** The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* 1991. **9:** 271–296.

21 **Arthur, J., Butterfield, L., Roth, M., Bui, L., Kiertscher, S., Lau, R., Dubinett, S., Glaspy, J., McBride, W. and Economou, J.,** A comparison of gene transfer methods in human dendritic cells. *Cancer Gene Ther.* 1997. **4:** 17–25.

22 **Kim, C., Prevette, T., Cormier, J., Overwijk, W., Roden, M., Restifo, N., Rosenberg, S. and Marincola, F.M.,** Dendritic cells infected with poxviruses encoding MART-1/Melan A sensitize T lymphocytes in vitro. *J. Immunother.* 1997. **20:** 276–286.

23 **Romani, N., Gruner, S., Brang, D., Kämpgen, E., Lenz, A., Trockenbacher, B., Konwalinka, G., Fritsch, P. O., Steinman, R. M. and Schuler, G.,** Proliferating dendritic cell progenitors in human blood. *J. Exp. Med.* 1994. **180:** 83–93.

24 **Sallusto, F. and Lanzavecchia, A.,** Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α. *J. Exp. Med.* 1994. **179:** 1109–1118.

25 **Jonuleit, H., Kuhn, U., Muller, G., Steinbrink, K., Paragnik, L., Schmitt, E., Knop, J. and Enk, A. H.,** Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *Eur. J. Immunol.* 1997. **27:** 3135–3142.

26 **Rammensee, H.-G., Bachmann, J. and Stevanovic, S.** (Eds.) *MHC Ligands and Peptide Motifs.* Springer, Berlin 1997.

27 **Traversari, C., van der Bruggen, P., Luescher, I. F., Lurquin, C., Chomez, P., van Pel, A., De Plaen, E., Amar-Costesec, A. and Boon, T.,** A nonapeptide encoded by human gene MAGE-1 is recognized on HLA-A1 by cytolytic T lymphocytes directed against tumor antigen MZ2-E. *J. Exp. Med.* 1992. **176:** 1453–1457.

28 **van der Bruggen, P., Szikora, J.-P., Boël, P., Wildmann, C., Somville, M., Sensi, M. and Boon, T.,** Autologous cytolytic T lymphocytes recognize a MAGE-1 nonapeptide on melanomas expressing HLA-Cw*1601. *Eur. J. Immunol.* 1994. **24:** 2134–2140.

29 **Huang, L.-Q., Brasseur, F., Serrano, A., De Plaen, E., van der Bruggen, P., Boon, T. and Van Pel, A.,** Cytolytic T lymphocytes recognize an antigen encoded by *MAGE-A10* on a human melanoma. *J. Immunol.* 1999. **162:** 6849–6854.

30 **Gaugler, B., Van den Eynde, B., van der Bruggen, P., Romero, P., Gaforio, J. J., De Plaen, E., Lethé, B., Brasseur, F. and Boon, T.,** Human gene MAGE-3 codes for an antigen recognized on a melanoma by autologous cytolytic T lymphocytes. *J. Exp. Med.* 1994. **179:** 921–930.

31 **Valmori, D., Gileadi, U., Servis, C., Dunbar, P. R., Cerottini, J.-C., Romero, P., Cerundolo, V. and Levy, F.,** Modulation of proteasomal activity required for the generation of a cytotoxic T lymphocyte-defined peptide derived from the tumor antigen MAGE-3. *J. Exp. Med.* 1999. **189:** 895–906.

32 **Takaki, T., Hiraki, A., Uenaka, A., Gomi, S., Itoh, K., Udono, H., Shibuya, A., Tsuji, T., Sekiguchi, S. and Nakayama, E.,** Variable expression on lung cancer cell lines of HLA-A2-binding MAGE-3 peptide recognized by cytotoxic T lymphocytes. *Int. J. Oncol.* 1998. **12:** 1103–1109.

33 **Tanaka, F., Fujie, T., Go, H., Baba, K., Mori, M., Takesako, K. and Akiyoshi, T.,** Efficient induction of antitumor cytotoxic T lymphocytes from a healthy donor using HLA-A2-restricted MAGE-3 peptide in vitro. *Cancer Immunol. Immunother.* 1997. **44:** 21–26.

34 **Shichijo, S., Hayashi, A., Takamori, S., Tsunosue, R., Hoshino, R., Sakata, M., Kuramoto, T., Oizumi, K. and itoh, K.,** Detection of MAGE-4 protein in lung cancers. *Int. J. Cancer* 1995. **64:** 158–165.

35 **Iwamoto, O., Nagao, Y., Shichijo, S., Eura, M., Kameyama, T. and Itoh, K.,** Detection of MAGE-4 protein in sera of patients with head-and-neck squamous-cell carcinoma. *Int. J. Cancer* 1997. **70:** 287–290.

36 **Altman, J. D., Moss, P. A. H., Goulder, P. J. R., Barouch, D. H., McHeyzer-Williams, M. G., Bell, J. I., McMichael, A. J. and Davis, M. M.,** Phenotypic analysis of antigen-specific T lymphocytes. *Science* 1996. **274:** 94–96.

37 **Romero, P., Rod Dunbar, P., Valmori, D., Pittet, M., Ogg, G. S., Rimoldi, D., Chen, J. L., Lienard, D., Cerottini, J. C. and Cerundolo, V.,** Ex vivo staining of metastatic lymph nodes by class I major histocompatibility complex tetramers reveals high numbers of antigen-experienced tumor-specific cytolytic T lymphocytes. *J. Exp. Med.* 1998. **188:** 1641–1650.

38 **Carney, D. N., Bunn, P. A., Gazdar, A. F., Pagan, J. A. and Minna, J. D.,** Selective growth in serum-free hormone-supplemented medium of tumor cells obtained by biopsy from patients with small cell carcinoma of the lung. *Proc. Natl. Acad. Sci. USA* 1981. **78:** 3185–3189.

39 **Stratford-Perricaudet, L. D., Makeh, I., Perricaudet, M. and Briand, P.,** Widespread long-term gene transfer to mouse skeletal muscles and heart. *J. Clin. Invest.* 1992. **90:** 626–630.

40 **Sory, M. P. and Cornelis, G. R.,** Translocation of a hybrid YopE-adenylate cyclase from Yersinia enterocolitica into HeLa cells. *Mol. Microbiol.* 1994. **14:** 583–594.

41 **Cornelis, G. R.,** The Yersinia deadly kiss. *J. Bacteriol.* 1998. **180:** 5495–5504.

42 **Boland, A. and Cornelis, G. R.,** Role of YopP in suppression of Tumor Necrosis Factor alpha release by macrophages during Yersinia infection. *Infect. Immun.* 1998. **66:** 1878–1884.

43 **Neyt, C., Iriarte, M., Ha Thi, V. and Cornelis, G. R.,** Virulence and arsenic resistance in Yersiniae. *J. Bacteriol.* 1997. **179:** 612–619.

44 **Traversari, C., van der Bruggen, P., Van den Eynde, B., Hainaut, P., Lemoine, C., Ohta, N., Old, L. and Boon, T.,** Transfection and expression of a gene coding for a human melanoma antigen recognized by autologous cytolytic T lymphocytes. *Immunogenetics* 1992. **35:** 145–152.

45 **Espevik, T. and Nissen-Meyer, J.,** A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. *J. Immunol. Methods* 1986. **95:** 99–105.

46 **Hansen, M. B., Nielsen, S. E. and Berg, K.,** Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J. Immunol. Methods* 1989. **119:** 203–210.

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A *MAGE-A4* peptide presented by HLA-B37 is recognized on human tumors by cytolytic T lymphocytes

Key words:
antigenic peptide; CTL; HLA-B37; *MAGE-A4*; tumor

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Abbreviations:
CTL: cytolytic T lymphocyte; EBV-B cells: Epstein-Barr virus-transformed B cells.

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Abstract: 'Cancer-germline' genes such as those of the *MAGE* family are expressed in many tumors and in male germline cells, but are silent in normal tissues. They encode shared tumor-specific antigens, which have been used in therapeutic vaccination trials of cancer patients. *MAGE-A4* is expressed in more than 50% of carcinomas of esophagus, head and neck, lung, and bladder. We report here the identification of a new *MAGE-A4* encoded peptide, which is recognized by a cytolytic T lymphocyte (CTL) clone on HLA-B*3701. The sequence of the peptide is SESLKMIF. It corresponds to the MAGE-$A4_{156-163}$ protein sequence. When tumor cells expressing *MAGE-A4* were transfected with HLA-B*3701, they were recognized by the CTL clone, demonstrating that the peptide ought to be processed in tumor cells and could therefore serve as a target for therapeutic antitumoral vaccination.

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MAGE-A4 belongs to the *MAGE* gene family which comprises 24 related functional genes divided into three clusters, named *MAGE-A, B,* and *C* (1–4). These genes are expressed in many human tumors of different histological types, but are silent in normal cells with the exception of testis. Some, including *MAGE-A4*, are also expressed in placenta (1). Male germline cells and placenta do not express MHC class I molecules and are therefore incapable of presenting antigens to cytolytic T lymphocyte (CTL) (5,6). *MAGE*-encoded antigens are thus tumor-specific, and are of particular interest for cancer immunotherapy because they are shared by many tumors. Clinical trials involving defined tumor-specific shared antigens have been and are being performed in melanoma patients, and tumor regressions have been observed in a minority of patients (7–10).

There are two known alleles of gene *MAGE-A4*, *MAGE-A4a* and *MAGE-A4b*. They code for proteins that differ by a single amino acid (11). The gene contains eight alternative first exons that are

spliced to unique second and third exons. The entire open reading frame is located in the third exon. The role of the different first exons, each with their own promoter, is unknown. We have used in this work the coding sequence of *MAGE-A4a*, referred to hereafter as *MAGE-A4*. *MAGE-A4* is expressed in a significant proportion of tumors (Table 1).

We reported previously the identification of peptide GVYDGREHTV (*MAGE-A4*$_{230-239}$), which is recognized by cytolytic T lymphocytes (CTL) on HLA-A2 (12). Only one antigenic peptide has been hitherto identified in the *MAGE-A4* protein. The identification of a large number of antigenic peptides presented by HLA class I and class II is likely to be important for the future of clinical trials with defined antigenic peptides. A large set of peptide/HLA combinations will alleviate HLA restriction and widen the set of eligible patients. It will also facilitate the design of concurrent immunizations against several antigens. Such immunizations could increase the primary antitumor efficacy of the vaccine and also decrease the risk of tumor escape by loss of antigen expression.

To isolate the first *MAGE-A4*-specific CTL, we had stimulated $CD8^+$ T cells from non-cancerous blood donors with autologous dendritic cells infected with an adenovirus carrying the coding sequence of *MAGE-A4*. Here, we have used a slightly different strategy: the dendritic stimulator cells were infected with an avian poxvirus, ALVAC, carrying the coding sequence of *MAGE-A4*. We describe here the identification of a new *MAGE-A4* antigen.

Materials and methods

Cell lines, media, and reagents

The Epstein–Barr Virus-transformed B (EBV-B) cell lines and the melanoma cell lines were cultured in IMDM supplemented with 10% FCS. 293-EBNA cells were maintained in DMEM supplemented with 10% FCS. All culture media were purchased from GibcoBRL (Paisley, UK) and supplemented with 0.24 mM of L-asparagine, 0.55 mM of L-arginine, 1.5 mM of L-glutamine (AAG), 100 U/ml of penicillin, and 100 μg/ml of streptomycin. Cell line MZ2-MEL 2.2-*MAGE-A4* was obtained by cotransfecting into MZ2-MEL 2.2 a pcDNAI/Amp plasmid (Invitrogen) that contains the coding sequence of *MAGE-A4* together with vector pSvtkneoβ, which contains the coding sequence conferring resistance to geneticin (13). Human recombinant IL-2 was purchased from Eurocetus (Amsterdam, the Netherlands). IL-7 was purchased from Genzyme (Cambridge, MA), GM-CSF (Leucomax) from Schering-Plough (Brinny, Ireland) and IFN-γ from Peprotech (Rocky Hill, USA). Human recombinant IL-4, IL-6, and IL-12 were produced in our laboratory. One U/ml of IL-6 is the concentration needed to obtain half-maximal proliferation of mouse 7TD1 cells (14). Geneticin was purchased from GibcoBRL.

Recombinant viruses and infection of cell lines

The recombinant canarypox ALVAC-*MAGE-A4*, the vaccinia-*MAGE-A4*, and the parental vaccinia viruses were provided by Aventis Pasteur (Lyon, France). Retroviral vector *MAGE-A4*-CSM encodes the full length *MAGE-A4* protein and the truncated form of the human low affinity nerve growth factor receptor (LNGFr). It was produced as previously reported (15). Epstein–Barr Virus-B cells were transduced by coculture with irradiated packaging cell lines producing the *MAGE-A4*-CSM vector in the presence of polybrene (8 μg/ml). After 72 h, lymphocytes were harvested and seeded in fresh medium. The percentage of infected cells was evaluated

Expression of gene MAGE-A4 by tumoral tissues*

Histological type	Percentage	$n^\dagger$
Bladder carcinoma		
superficial (<T2)	23	(70)
infiltrating (=T2)	45	(53)
Breast carcinoma	6	(135)
Colorectal carcinoma	11	(46)
Esophageal squamous-cell carcinoma	74	(19)
Head & neck squamous-cell carcinoma	53	(85)
Hepatocellular carcinoma	16	(50)
Leukemia	1	(112)
Lung carcinoma		
squamous-cell carcinoma	59	(93)
adenocarcinoma	35	(43)
Melanoma		
primary lesions	18	(83)
metastases	28	(243)
Myeloma		
stages I–II	0	(11)
stage III	22	(27)
Pediatric rhabdomyosarcoma	22	(31)
Prostate carcinoma	0	(22)
Renal cell carcinoma	2	(44)
Sarcoma	33	(15)

*Expression was measured by RT-PCR on total RNA of surgical samples using a primer specific for *MAGE-A4*. All results were obtained by Francis Brasseur at the Brussels Branch of the Ludwig Institute for Cancer Research(25), except for hepatocellular carcinoma(26) and pediatric rhabdomyosarcoma(27).
†Number of tumors tested.

Table 1

48h later by flow cytometry for LNGFr expression with the mAb 20.4 (ATCC, Rockville, MD). The LNGFr-positive cells were purified by magnetic cell sorting using rat antimouse IgG1-coated beads (Dynabeads M-450, DYNAL A.S. N012 Oslo, Norway). Using the same protocol, melanoma cell lines AVL3-MEL and LB1751-MEL were transduced with retroviral vector B37-CSM, which codes for the HLA-B*3701 molecule and the truncated form of LNGFr.

Dendritic cells and CD8$^+$ responder T cells

Peripheral blood was obtained from hemochromatosis patient LB2257 as standard buffy coat preparations, which were laid down on a 15-ml Lymphoprep layer (Axis-Shield PoCAS, Oslo, Norway) in 50-ml tubes. To minimize contamination of the blood cells by platelets, the tubes were first centrifuged at 200g for 20min at room temperature. After removal of the top 20–25ml, containing most of the platelets, the tubes were centrifuged at 300g for 20min at room temperature. The interphase containing the PBMCs was harvested and washed three times (or more) in cold phosphate buffer solution with 2mM of EDTA in order to eliminate the remaining platelets. To generate autologous dendritic cells, the PBMCs were depleted from the T lymphocytes by rosetting with sheep erythrocytes (Bio Mérieux, Marcy-l'Etoile, France) treated with 2-aminoethylisothiouronium (Sigma-Aldrich, Steinheim, Germany). Rosetted T cells were treated with NH_4CL (160mM) to lyze the sheep erythrocytes and washed. CD8$^+$ T lymphocytes were isolated from the rosetted T cells by positive selection using an anti-CD8 mAb coupled to magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). They were then sorted through a magnet and subsequently frozen. The day before the coculture with the dendritic cells, the CD8$^+$ T cells were thawed and grown overnight in IMDM supplemented with 10% human serum, AAG, and antibiotics (hereafter referred to as complete IMDM) in the presence of 5U/ml of IL-2. The lymphocyte-depleted PBMCs were left to adhere for 1h at 37°C in culture flasks (FALCON, Becton Dickinson) at a density of 10^6 cells per cm^2 in RPMI 1640 supplemented with Hepes (2.38g/l), AAG, antibiotics, and 10% FCS (hereafter referred to as complete RPMI medium). Nonadherent cells were discarded and the adherent cells were cultured in the presence of IL-4 (200U/ml) and GM-CSF (70ng/ml) in complete RPMI medium. Cultures were fed on days 2 and 4 by removing one-third of the volume and adding fresh medium with IL-4 (200U/ml) and GM-CSF (70ng/ml). They were frozen on day 6.

Mixed lymphocyte-dendritic cell culture

Dendritic cells (4×10^6) were infected with ALVAC-*MAGE-A4* at a multiplicity of infection (MOI) of 30 in 200µl of complete RPMI medium at 37°C under 5% CO_2. The infected dendritic cells were washed after 2h. Autologous responder CD8$^+$ T lymphocytes (1.5×10^5) were mixed with infected dendritic cells (3×10^4) in U-bottomed microwells in 200µl of complete IMDM in the presence of IL-6 (1000U/ml) and IL-12 (10ng/ml). On days 6 and 13, autologous dendritic cells were thawed and infected the day after with ALVAC-*MAGE-A4*. The infected cells were used to restimulate the responder lymphocytes in medium supplemented with IL-2 (10U/ml) and IL-7 (5ng/ml). The responder lymphocytes were assessed on day 21 for their capacity to lyze autologous EBV-B cells infected with vaccinia-*MAGE-A4*.

Cytotoxicity assay

The cytotoxicity of an aliquot of each microculture was tested on autologous EBV-B cells infected with either vaccinia-*MAGE-A4* or the parental vaccinia virus. Infection was performed on 2×10^6 target cells for 2h at an MOI of 20 in 150µl of complete RPMI medium. Infected cells were washed, labeled with 100µCi of $Na(^{51}Cr)O_4$, and were added to the responder cells at an effector/target (E:T) ratio of 40:1. Unlabeled K562 cells were also added (5×10^4 per V-bottom microwell) to block natural killer activity. Individual microcultures were tested on each target in duplicate. Chromium release was measured after incubation at 37°C for 4h. The melanoma cell lines were labeled with $Na(^{51}Cr)O_4$ as described earlier, and pulsed, if indicated, for 15min with 1µg/ml of peptide.

Isolation of CD8$^+$ T-cell clone specific for *MAGE-A4*

T cells from the microculture with anti-*MAGE-A4* reactivity were cloned in U-bottomed microplates by limiting dilution in complete IMDM supplemented with IL-2 (50U/ml) and 15µg/ml of gentamicin using, as stimulators, irradiated (100 Gy) $5–15\times10^3$ autologous EBV-B cells transduced with a retrovirus coding for *MAGE-A4*. Allogeneic EBV-B cells ($5–15\times10^3$ irradiated LG2-EBV-B cells per well) were used as feeder cells. Established CTL clones were grown in complete IMDM supplemented with IL-2 (50U/ml) and 3×10^5 CTL were passaged weekly with irradiated feeder cells (1.5×10^6 LG2 EBV-B cells per well in a 24-well plate) and irradiated autologous EBV-B cells were transduced with retrovirus coding for *MAGE-A4* (10^5 cells per well).

Transfection of 293-EBNA cells and TNF assay

293-EBNA cells (2×10^4) were distributed in flat-bottom microwells and cotransfected with pcDNAI/Amp containing the *MAGE-A4* cDNA (50ng) and 50ng of pcDNAI/Amp containing the coding se-

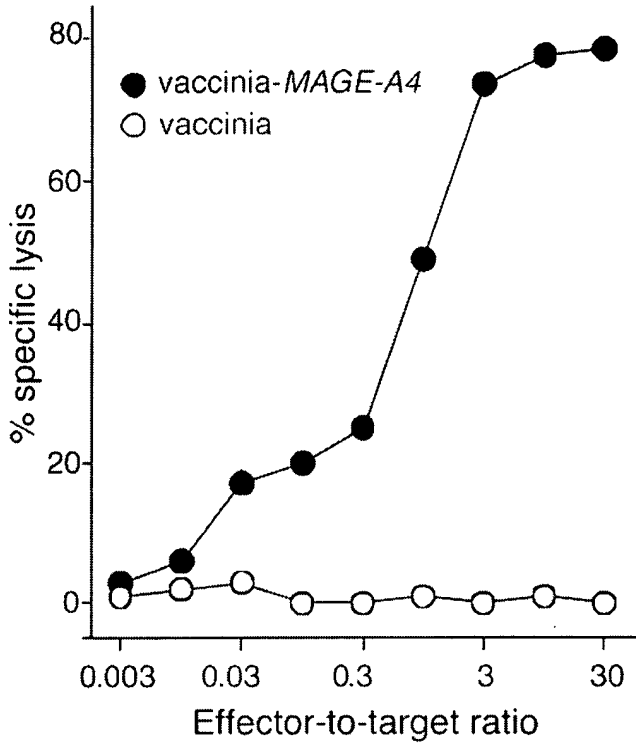

Fig. 1. **Lysis by cytolytic T lymphocyte F2 of autologous EBV-B cells infected with vaccinia-*MAGE-A4*.** Epstein–Barr Virus-B cells from donor LB2257 were infected for 2 h with the vaccinia vectors at a multiplicity of infection of 20, ^{51}Cr-labeled for 1 h, and incubated with autologous CTL F2 at the indicated effector-to-target ratios. Chromium release was measured after 4 h.

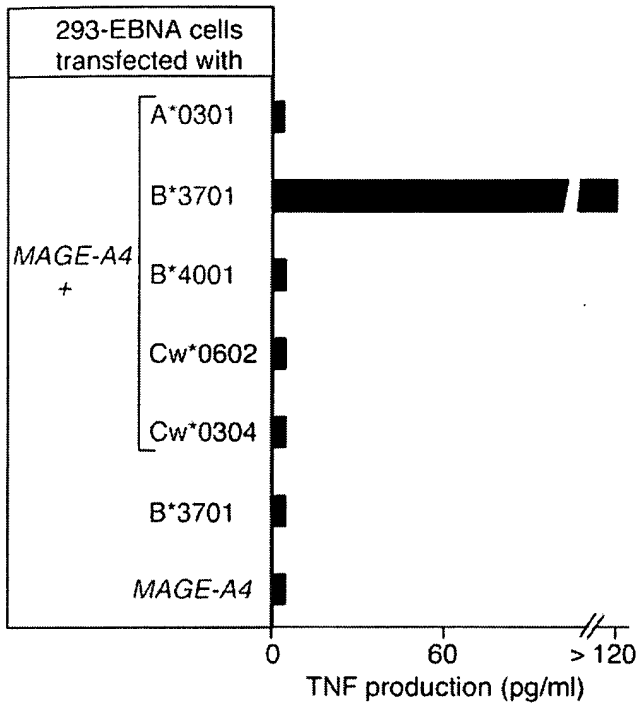

Fig. 2. **The *MAGE-A4* antigenic peptide is presented by HLA-B37 molecules. 293-EBNA cells were transiently transfected with a *MAGE-A4* cDNA and each of the cDNAs coding for the putative HLA-presenting molecules.** The cDNAs were inserted into expression vector pcDNAI/Amp. Transfections were performed in microwells with 20,000 293-EBNA cells, 50 ng of each cDNA and 1 µl of Lipofectamine. One day after transfection, 5000 cytolytic T lymphocyte F2 were added to the transfected cells. TNF production was measured after overnight coculture by testing the toxicity of the supernatants for TNF-sensitive WEHI 164 clone 13 cells.

quences of each of the five putative HLA alleles using 1 µl of Lipofectamine (GibcoBRL). Transfected cells were incubated for 24 h at 37°C and 8% CO_2. The transfectants were then tested for their ability to stimulate the production of TNF by the CTL clone. Briefly, 5000 CTL were added to the microwells containing the transfectants, in a total volume of 100 µl of complete IMDM supplemented with 25 U/ml of IL-2. After 24 h, the supernatant was collected and its TNF content was determined by testing its cytotoxic effect on WEHI-164 clone 13 cells in a MTT colorimetric assay (13,16,17).

Peptide recognition assay

Peptides were synthesized on solid phase using Fmoc for transient NH2-terminal protection and were characterized using mass spectrometry. All peptides were >90% pure, as indicated by analytic HPLC. Lyophilized peptides were dissolved at 2 mg/ml in 10 mM of acetic acid and 10% DMSO, and were stored at −20°C. The first screening was performed with autologous EBV-B cells incubated with 16 amino-acid long peptides at a concentration of 1 µg/ml. Peptide-pulsed targets were tested for recognition by CTL at an E:T ratio of 5:1.

Results and discussion

Isolation of an anti-*MAGE-A4* CTL clone

Monocyte-derived dendritic cells of blood donor LB2257 were infected with an avian poxvirus, ALVAC, carrying the *MAGE-A4* coding sequence (ALVAC-*MAGE-A4*). These antigen-presenting cells were distributed in 96 microwells and were used to stimulate autologous $CD8^+$ T lymphocytes in the presence of IL-6 and IL-12. After two weekly restimulations with dendritic cells infected with ALVAC-

MAGE-A4 in the presence of IL-2 and IL-7, the responder cells were tested on day 21 for their specific lytic activity on the autologous EBV-B cells infected with a vaccinia poxvirus encoding *MAGE-A4* (vaccinia-*MAGE-A4*). The reason for using vaccinia instead of ALVAC was that EBV-B cells infected with ALVAC are poor targets for cytotoxicity assays (data not shown). EBV-B cells infected with the parental vaccinia were used as control targets because CTL can recognize antigens shared by ALVAC and vaccinia poxvirus (18).

One microculture with anti-*MAGE-A4* lytic activity was obtained. The responder lymphocytes were cloned by limiting dilution using, as stimulator cells, autologous EBV-B cells transduced with a retrovirus carrying the coding sequence of *MAGE-A4*. This new stimulation combination was used to stimulate the anti-*MAGE-A4* CTL, while avoiding restimulating CTL directed against poxvirus antigens. We obtained stable CTL clone LB2257 661/F2 (hereafter referred to as CTL F2), which lyzed the EBV-B cells infected with vaccinia-*MAGE-A4* but not the EBV-B cells infected with the parental vaccinia (Fig. 1).

Identification of the HLA presenting molecule

Blood donor LB2257 was typed HLA-A*0301, B*3701, B*4001, Cw*0302 and Cw*0602. To identify the HLA molecule that presents the *MAGE-A4* peptide recognized by CTL F2, 293-EBNA cells, which derived from a human embryonic kidney cell line, were transiently transfected with a *MAGE-A4* cDNA together with each of the cDNAs encoding the putative HLA presenting molecules. Only those cells that were transfected with *MAGE-A4* and HLA-B*3701 stimulated CTL F2 to produce TNF (Fig. 2).

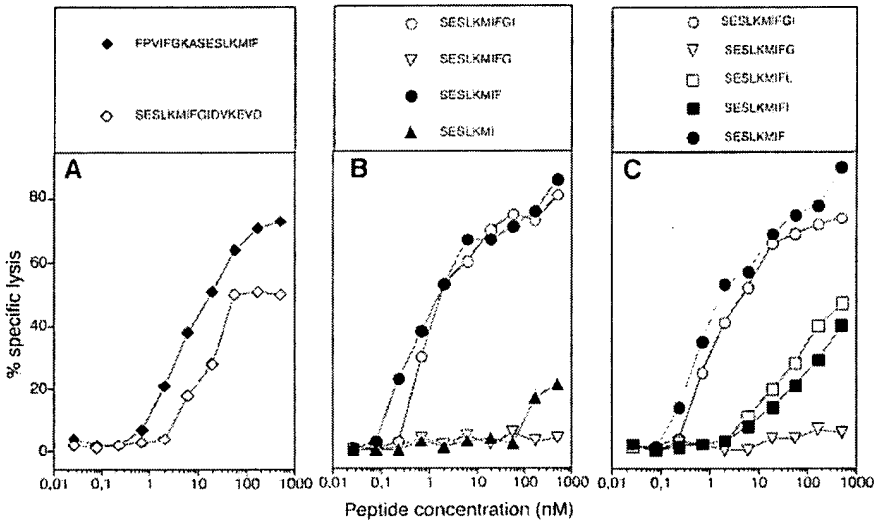

Fig. 3. **Peptide titration.** (*A*) Identification of two 16-mer peptides recognized by cytolytic T lymphocyte (CTL) F2. (*B*) Identification of the shortest peptide recognized by CTL F2. (*C*) Test of peptides modified at the C-terminus. Autologous EBV-B cells were ^{51}Cr-labeled and incubated for 15 min with threefold dilutions of synthetic peptides. Autologous CTL F2 was subsequently added at an effector-to-target ratio of 5:1. Chromium release was measured after 4 h. The concentrations indicated in the figure are the concentrations during 4-h incubation. Experiments described in (B) and (C) were performed with HPLC-purified peptides (99% pure).

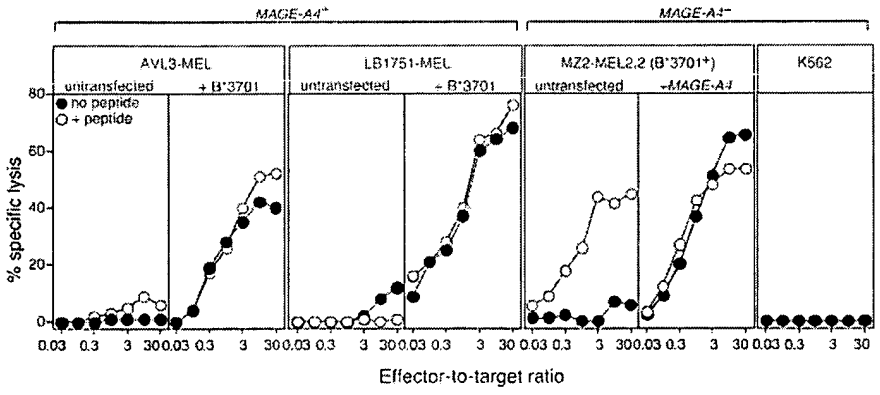

Fig. 4. **Lysis by cytolytic T lymphocyte F2 of B*3701 tumor cell lines expressing *MAGE-A4*.** Target cells were ^{51}Cr-labeled and, if indicated, pulsed for 15 min with 1 μg/ml of peptide SESLKMIF. They were then incubated for 4 h with CTL F2 at the indicated effector-to-target ratios. Chromium release was measured after 4 h.

Identification of the antigenic peptide

To determine the peptide recognized by CTL F2, we screened a set of peptides covering the entire *MAGE-A4* protein sequence. These 16 amino-acid long peptides were overlapped by 12 residues. Autologous EBV-B cells were incubated with 1 μg/ml of each of these peptides and were tested for lysis by CTL F2. Peptide FPVIFGKASESLKMIF (*MAGE-A4*$_{148-163}$) and SESLKMIFGIDVKEVD (*MAGE-A4*$_{156-171}$) scored positive (Fig. 3A). The consensus anchor residues for HLA-B37 are D/E in position 2, F/M/L in position 8, and I/L in the C-terminal position (19,20). One peptide, SESLKMIFGI, contains these three anchor residues but its sequence was only included in one of the two long peptides that scored positive. We therefore tested a number of peptides of different lengths. Peptide SESLKMIFGI and peptide SESLKMIF scored positive and half maximal lysis of autologous EBV-B target cells was obtained at a peptide concentration of 1 nM (Fig. 3B). This is within the range of the previously identified MAGE antigenic peptides, for which values ranging from 0.05 to 100 nM were observed (18,21–24). These two peptides are encoded by both *MAGE-A4a* and *MAGE-A4b*.

Interestingly, nonapeptide SESLKMIFG was not recognized by CTL F2 (Fig. 3B). The C-terminal residue G was replaced by a consensus anchor residue for B37, either a I or a L (19). Targets pulsed with a very high and most probably not physiological concentration of these two modified peptides were recognized by the CTL (Fig. 3C). The results suggest that the C-terminal residue is very important for the binding to B37 and that the presence of a G decreases the affinity of the peptide for B37.

Lysis of tumor cell lines

As we used dendritic cells expressing *MAGE-A4* to activate CTL F2, it was important to verify that tumor cells also process the *MAGE-A4* antigen. Because we had no tumor cell lines expressing both *MAGE-A4* and B*3701, we transduced *MAGE-A4*-expressing melanoma cell lines with a retrovirus carrying the B*3701 coding sequence. The B*3701-transduced cell lines were both lyzed by CTL F2 (Fig. 4). Melanoma cell line MZ2-MEL.2.2, which expressed B*3701 but not *MAGE-A4*, was transduced with a retrovirus carrying the *MAGE-A4* coding sequence. It was efficiently lyzed after transduction (Fig. 4). Control K562 cells were not lyzed.

The new *MAGE-A4* antigenic peptide described here is presented by B*3701 molecules. The HLA allelic group B37 comprises two alleles. B*3701 corresponds to the B37 serological specificity, but B*3702 is not recognized by serum antibodies and it is therefore considered as 'blank'. B37 molecules are rarely expressed in the different major ethnic groups: Black (2%), Caucasoid (3%), Oriental (2%), Amerindian (4%). Identification of additional *MAGE-A4* antigenic peptides will be important because a number of tumors express *MAGE-A4* without expressing MAGE-A1 and MAGE-A3. It is the case for carcinomas of the lung (15%), the head and neck (14%), the esophagus (11%), and the bladder (10%).

References

1. De Plaen E, Arden K, Traversari C et al. Structure, chromosomal localization and expression of twelve genes of the MAGE family. *Immunogenetics* 1994: **40**: 360–9.
2. Lurquin C, De Smet C, Brasseur F et al. Two members of the human *MAGEB* gene family located in Xp.21.3 are expressed in tumors of various histological origins. *Genomics* 1997: **46**: 397–408.
3. Lucas S, De Smet C, Arden KC et al. Identification of a new MAGE gene with tumor-specific expression by representational difference analysis. *Cancer Res* 1998: **58**: 743–52.
4. Chomez P, De Backer O, Bertrand M, De Plaen E, Boon T, Lucas S. An overview of the MAGE gene family with the identification of all human members of the family. *Cancer Res* 2001: **61**: 5544–51.
5. Haas GG Jr, D'Cruz OJ, De Bault LE. Distribution of human leukocyte antigen-ABC and – D/DR antigens in the unfixed human testis. *Am J Reprod Immunol Microbiol* 1988: **18**: 47–51.
6. Hunt DF, Henderson RA, Shabanowitz J et al. Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. *Science* 1992: **255**: 1261–3.
7. Marchand M, Brichard V, van Baren N, Coulie PG. Biological and clinical developments in melanoma vaccines. *Exp Opin Biol Ther* 2001: **1**: 497–510.
8. Jäger E, Jäger D, Knuth A. Clinical cancer vaccine trials. *Curr Opin Immunol* 2002: **14**: 178–82.
9. Marchand M, van Baren N, Weynants P et al. Tumor regressions observed in patients with metastatic melanoma treated with an antigenic peptide encoded by gene *MAGE-3* and presented by HLA-A1. *Int J Cancer* 1999: **80**: 219–30.
10. Thurner B, Haendle I, Roder C et al. Vaccination with MAGE-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. *J Exp Med* 1999: **190**: 1669–78.
11. De Plaen E, Naerhuyzen B, De Smet C, Szikora J-P, Boon T. Alternative promoters of gene MAGE4a. *Genomics* 1997: **40**: 305–13.
12. Duffour M-T, Chaux P, Lurquin C, Cornelis G, Boon T, van der Bruggen P. A MAGE-A4 peptide presented by HLA-A2 is recognized by cytolytic T lymphocytes. *Eur J Immunol* 1999: **29**: 3329–37.

13. Traversari C, van der Bruggen P, Van den Eynde B et al. Transfection and expression of a gene coding for a human melanoma antigen recognized by autologous cytolytic T lymphocytes. *Immunogenetics* 1992: **35**: 145–52.
14. Van Snick J, Vink A, Cayphas S, Uyttenhove C. Interleukin-HP1, a T cell-derived hybridoma growth factor that supports the in vitro growth of murine plasmocytomas. *J Exp Med* 1987: **165**: 641–9.
15. Mavilio F, Ferrari G, Rossini S et al. Peripheral blood lymphocytes as target cells of retroviral vector-mediated gene transfer. *Blood* 1994: **83**: 1988–97.
16. Espevik T, Nissen-Meyer J. A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. *J Immunol Meth* 1986: **95**: 99–105.
17. Hansen MB, Nielsen SE, Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J Immunol Meth* 1989: **119**: 203–10.
18. Chaux P, Luiten R, Demotte N et al. Identification of five MAGE-A1 epitopes recognized by cytolytic T lymphocytes obtained by *in vitro* stimulation with dendritic cells transduced with. *MAGE-A1 J Immunol* 1999: **163**: 2928–36.
19. Rammensee H-G, Bachmann J, Stevanovic S. *MHC Ligands and Peptide Motifs*. New York: Springer, 1997.
20. Falk K, Rötzschke O, Grahovac B et al. Peptide motifs of HLA-B35 and – B37 molecules. *Immunogenetics* 1993: **38**: 161–2.
21. Schultz ES, Zhang Y, Knowles R et al. A MAGE-3 peptide recognized on HLA-B35 and HLA-A1 by cytolytic T lymphocytes. *Tissue Antigens* 2001: **57**: 103–9.
22. Luiten R, van der Bruggen P. A MAGE-A1 peptide is recognized on HLA-B7 human tumors by cytolytic T lymphocytes. *Tissue Antigens* 2000: **55**: 149–52.
23. Traversari C, van der Bruggen P, Luescher IF et al. A nonapeptide encoded by human gene MAGE-1 is recognized on HLA-A1 by cytolytic T lymphocytes directed against tumor antigen MZ2-E. *J Exp Med* 1992: **176**: 1453–7.
24. van der Bruggen P, Szikora J-P, Boël P et al. Autologous cytolytic T lymphocytes recognize a MAGE-1 nonapeptide on melanomas expressing HLA-Cw*1601. *Eur J Immunol* 1994; **24**: 2134–40.
25. van der Bruggen P, Zhang Y, Chaux P et al. Tumor-specific shared antigenic peptides recognized by human T cells. *Immunol Rev*, in. press.
26. Chen CH, Huang GT, Lee HS et al. High frequency of expression of MAGE genes in human hepatocellular carcinoma. *Liver* 1999: **19**: 110–4.
27. Dalerba P, Frascella E, Macino B et al. MAGE, BAGE and GAGE gene expression in human rhabdomyosarcomas. *Int J Cancer* 2001: **93**: 85–90.